Neuropeptide regulation of hormone secretion from the anterior pituitary gland of the ewe

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Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

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Abstract

The actions of LHRH, CRH and NPY on the activity of the reproductive axis and, in particular, the LHRH pulse generator were investigated *in vivo*. A model was developed to allow direct administration of neuropeptides, antagonists and antibodies into the third cerebral ventricle of conscious, free-moving and unstressed sheep, giving access to hypothalamic periventricular structures involved in the control of the anterior pituitary gland. Plasma levels of LH were used as a measure of LHRH pulsatility. Three major questions were addressed:

(1) Does hypothalamic LHRH autoregulate its own release? Injection of LHRH (2.1–21 pmol) into the third ventricle caused a specific, dose-related and receptor-mediated inhibition of LH secretion. It is therefore suggested that the intrinsic communication between LHRH neurones may be inhibitory and may provide a mechanism to affect the timing of the pulsatile release of LHRH. However, as central injection of LHRH also caused a rapid 4–5-fold rise in plasma cortisol levels, prior to and correlated with the reduction in LH secretion, this inhibitory communication may be indirect.

(2) Does CRH act as a central neurotransmitter to mediate the inhibitory effects of stress on LHRH release? In contrast to other species, central injection of CRH (0.12–1.2 nmol) caused a dose-related stimulation of LH secretion, due to a significant increase in LH pulse frequency. CRH also caused a marked and dose-related stimulation of prolactin and cortisol secretion, two hormones known to be released under conditions of stress. Endogenous opioid peptides were shown to mediate the central effect of CRH on the release of prolactin, but not on LH or cortisol. The effect on LH may reflect a species difference, or alternatively may be similar to the increase in LH secretion reported in rats and monkeys subjected to short-term handling or restraint stress. These data provide evidence that CRH acts as a central neurotransmitter; i.e., distinct from its action as a secretagogue for ACTH.

(3) Does central NPY interact with LHRH and/or CRH to modulate the reproductive axis? In contrast to the rat, central injection of NPY (0.15–1.5 nmol) had no effect on LH levels in ovariectomized ewes with or

without oestradiol implants; nor was LH secretion altered by central NPY in intact animals. However, central administration of NPY (1.5 nmol) during both the follicular and luteal phases, and in the oestradiol-implanted ovariectomized ewe, caused a large and significant increase in plasma cortisol. Passive systemic immunization with high-titre antibodies raised against NPY had no effect on LH secretion during either the oestradiol-induced LH surge in anoestrous ewes or the pre-ovulatory LH surge in intact ewes. Central administration of anti-NPY antibodies, however, resulted in a delay in the onset of the pre-ovulatory LH surge. These results demonstrate that NPY could play a part in the modulation of the timing of the LH surge at the level of the hypothalamus and also that NPY is involved in the multi-factorial regulation of ACTH release.

Thus complex and hierarchical intra-hypothalamic interactions exist between neuropeptides involved in the control of the anterior pituitary gland of the ewe: (1) LHRH regulates the pattern of its own release via a hypothalamic mechanism; (2) CRH acts as a central transmitter integrating neuroendocrine responses to stress; (3) NPY modulates the timing of the pre-ovulatory LH surge and the activity of the hypothalamo-pituitary-adrenal axis.

[~42 000 words in main text]

Abbreviations

Ab	antibody, antibodies
ACTH	adrenocorticotrophic hormone, corticotrophin
AHA	anterior hypothalamic area
ANF	atrial natriuretic factor
AVP	arginine vasopressin
BSA	bovine serum albumin
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CPON	C-terminal flanking peptide of NPY
CRF	corticotrophin-releasing factor
CRH	corticotrophin-releasing hormone
CSF	cerebrospinal fluid
CV	coefficient of variation
E_2	oestradiol
E_2B	oestradiol benzoate
EOP	endogenous opioid peptide
FSH	follicle-stimulating hormone
FSH-RF	follicle-stimulating hormone-releasing factor
GABA	γ-aminobutyric acid
GAP	GnRH-associated peptide
GH	growth hormone
GnRH	gonadotrophin-releasing hormone, LHRH
h	human, as in hNPY
HPA	hypothalamo-pituitary-adrenal
hpg	hypogonadal [mouse]
i.c.v.	intracerebroventricular(ly)
i.m.	intramuscular(ly)
ir	immunoreactive, immunoreactivity (as in ir-NPY, NPY-ir)
IRMA	immunoradiometric assay
i.v.	intravenous(ly)
K_d	dissociation constant
LH	luteinizing hormone

LHRH	luteinizing hormone-releasing hormone
MBH	mediobasal hypothalamus
ME	median eminence
mRNA	messenger ribonucleic acid
MUA	multi-unit activity
NIH	National Institutes of Health
NPY	neuropeptide Y, neuropeptide tyrosine
o	ovine (as in oCRH)
OVLT	organum vasculosum lamina terminalis
OVX	ovariectomized
OVX/E_2	ovariectomized [ewes] implanted with oestradiol
p	porcine (as in pPP)
PACAP	pituitary adenylate cyclase-activating polypeptide
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PHI	peptide histidine-isoleucine
PIF	prolactin-inhibiting factor
PNS	peripheral nervous system
POA	pre-optic area
PP	pancreatic polypeptide
pTG	porcine thyroglobulin
PVN	paraventricular nucleus of the hypothalamus
PYY	peptide YY, peptide tyrosine-tyrosine
rγG	rabbit γ-globulin
RIA	radioimmunoassay
SAPU	Scottish Antibody Production Unit
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus
VIP	vasoactive intestinal polypeptide

1. Introduction

Reproduction in female mammals is regulated by complex interactions between neuropeptides, glycoprotein hormones and steroids secreted from the hypothalamus, the anterior pituitary gland and the ovary, respectively. Luteinizing hormone-releasing hormone (LHRH) is released from the hypothalamus into the pituitary portal circulation, where it passes to the anterior pituitary gland and triggers the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These, in turn, control the functions of the ovary. Steroids released from the ovary feed back on the pituitary and hypothalamus to modulate the secretion of LHRH and the gonadotrophins. Superimposed upon this primary control are important external influences such as photoperiod, stress, olfactory and social cues.

Since its isolation, characterization and subsequent synthesis by the research groups of Andrew Schally and Roger Guillemin almost 20 years ago (Matsuo, Arimura, Nair & Schally, 1971*a*; Burgus, Butcher, Amoss *et al.* 1972), the hypothalamic decapeptide LHRH has been shown to be the single most important neuropeptide in reproduction, governing—directly or indirectly—almost every reproductive process. However, it is also clear that many other neuropeptides have the ability to regulate or modulate the secretion of LHRH and therefore the secretion of LH.

In the last 10–15 years there has been a surge of knowledge in the neuropeptide field. Immunocytochemical studies have established that many biologically-active peptides exist widely throughout the central nervous system. Many of these peptides had already been isolated from other tissues, particularly the gut. Examples include cholecystokinin (CCK), substance P and vasoactive intestinal polypeptide (VIP). In other cases, hypothalamic peptides known to serve as releasing or inhibiting factors for pituitary hormones (e.g. somatostatin and LHRH) were found to have an independent existence elsewhere in the CNS. This widespread distribution of peptides within the brain suggests that these substances may function under physiological conditions as neurotransmitters or neuromodulators.

The 'classical' role of the hypothalamic releasing factors in the control of pituitary hormone secretion has been recognized for many years, but it

has only recently become clear that neuropeptides can have many other actions both within the hypothalamus and elsewhere in the brain. For example, LHRH in the limbic system has a role in behavioural and mood changes; vasopressin acts as a neurotransmitter involved in the thermoregulatory responses to fever and also seems to be involved in memory processes; and CCK-8, as well as its well-documented role in the inhibition of feeding, has recently been suggested to have a role as a transmitter involved in mediating anxiety.

Neuropeptides differ from the classical neurotransmitters in that they are synthesized as large protein precursors in the perikarya of neurones, undergo post-translational processing by proteolytic cleavage, glycosylation and/or acylation and are transported to the nerve terminals packaged in vesicles. After release, peptides must be degraded back to their constituent amino acids as cellular uptake of intact peptides for recycling apparently does not occur. This is reflected in the longer-acting, modulatory effects of many neuropeptides. In contrast, the 'classical' neurotransmitters are largely synthesized in nerve terminals from appropriate precursors and can be taken up into presynaptic terminals by specific uptake systems (e.g. noradrenaline) or broken down by enzyme action (e.g. acetylcholine), thus permitting rapid recycling of active chemicals. The secretion and synthesis of neuropeptides is therefore metabolically expensive when compared with the 'classical' neurotransmitters. However, this is offset by a much higher potency and specificity, which allows for effective actions from the release of much smaller quantities of neuropeptide and for action at remote sites via transport in extracellular fluid, cerebrospinal fluid (CSF) and plasma.

In this thesis, experiments are described to investigate aspects of the physiology of hypothalamo-pituitary interactions *in vivo*, with reference to the effects of the hypothalamic neuropeptides LHRH, corticotrophin-releasing hormone (CRH) and neuropeptide Y (NPY) on the activity of the reproductive axis and, in particular, the LHRH pulse generator. A model was developed, using sheep, that allowed the administration of neuropeptides, antagonists and antibodies directly into the third cerebral ventricle. The third ventricle is a midline structure, filled with CSF. It bathes the hypothalamus on either side and below, therefore allowing access by diffusion to periventricular structures involved in the control of the anterior pituitary such as the paraventricular nucleus of the hypothalamus (PVN), the mediobasal hypothalamus (MBH), the preoptic

area (POA), the anterior hypothalamic area (AHA), and the arcuate (or infundibular) nucleus. Plasma levels of LH were used to assess the secretion of LHRH. Also of interest were the effects of LHRH, CRH and NPY on the secretion of FSH, prolactin and adrenocorticotrophic hormone (corticotrophin; ACTH) from the anterior pituitary gland. However, as ACTH-immunoreactivity is affected adversely by freeze-thaw cycles, plasma cortisol levels were determined as a measure of pituitary ACTH secretion. The main advantages of this model are that it allows experiments in conscious, free-moving and unstressed animals, whilst serial samples can be taken to allow the monitoring of pulsatile hormone secretion over long periods of time as the sheep has a large blood volume and a particularly well-characterized pattern of LH release. Details of the animals, surgical procedures, assays and other general methods are given in Chapter 3.

Three major areas were addressed in the experiments reported in this thesis. The first centred on the concept of LHRH autoregulation, or 'ultra-short'-loop feedback and potential mechanisms whereby this might be achieved (Chapters 4–6). The second theme involved the role of CRH as a neurotransmitter mediating the inhibitory effects of stress on the secretion and/or release of LHRH (Chapter 7). The third area of investigation was the interactive role of the recently-discovered hypothalamic peptide NPY in the modulation of the reproductive and hypothalamo-pituitary-adrenal axes (Chapters 8 and 9).

Each chapter has a specific introductory section relevant to the questions addressed by those experiments, and aims to review the work of others in that particular field. Thus, the overview provided in Chapter 2 does not set out to review the whole literature pertinent to all the material in the thesis: it aims solely to provide a foundation for the understanding and appreciation of the studies reported in the rest of the thesis. Chapter 2, therefore, contains background information on the neuroendocrine physiology of LHRH; aspects of the hypothalamic control of the stress axis are addressed briefly; and a detailed review of selected aspects of the biology of neuropeptide Y is given. Each experimental chapter concludes with a discussion of the results in the context of those of other investigators. Limitations of the approaches used in the thesis, questions that remain, and areas ripe for further investigation are discussed in Chapter 10, with suggestions as to how these might be addressed.

2. Background

2.1. LHRH and the control of reproduction in the ewe

The reproductive cycle of the ewe is seasonal, comprising periods of reproductive cyclicity (the breeding season) and reproductive quiescence (anoestrus) each lasting approximately 6 months, depending on the breed (for reviews see Karsch, 1980; Lincoln & Short, 1980). The breeding season is confined to autumn and winter, which ensures that the lambs are born in the favourable environment of spring. It consists of successive 16–17 day oestrous cycles, unless fertilization and pregnancy supervene. Each cycle starts with a period of sexual receptivity (oestrus) followed by ovulation, and is characterized by distinctive variations in the concentrations of reproductive hormones in the plasma.

As will become clear, the primary control of reproduction depends upon the secretion of LHRH—and possibly upon the secretion of a still-putative FSH-releasing factor (FSH-RF)—from the hypothalamus. Under the influence of LHRH, LH (and FSH) is secreted from the anterior pituitary gland. The pattern of LH release comprises a tonic secretion of LH which is interrupted once every cycle by a massive discharge of LH after oestrus (called the pre-ovulatory LH surge), which allows the animal to ovulate. This surge is governed by changes in the characteristics of the feedback mechanisms of ovarian steroids. The tonic mode of LH secretion is necessary for normal development of the ovaries and for the secretion of both oestradiol from large ovarian follicles and progesterone from the corpus luteum (the temporary endocrine gland formed from the remnants of a follicle after it has ovulated). Tonic LH secretion, in turn, is regulated by the feedback effects of these ovarian steroids. The longest portion of the sheep oestrous cycle (~80%) is the luteal phase, during which the corpus luteum develops and secretes large quantities of progesterone. In the remainder of the cycle, the 2–3 day follicular phase, the corpus luteum regresses, resulting in a precipitous drop in circulating progesterone, while the follicle(s) destined to ovulate next enlarge and secrete an increasing quantity of oestradiol. This phase culminates in the pre-ovulatory surge

of LH and the consequent ovulation of the follicle.

2.1.1. Demonstration of pulsatile LH secretion

Prior to the development and application of sensitive radioimmunoassay (RIA) techniques, the large increase in LH at the time of the surge was the only detectable change throughout the sheep oestrous cycle. Even this could only be detected using cumbersome bioassay techniques such as the ovarian ascorbic acid depletion method (e.g., see Dierschke & Clegg, 1968). With the development of more sensitive bioassays and the advent of RIA in the late 1950s (Yalow & Berson, 1959), however, it had become possible to measure concentrations of selected hormones in relatively small amounts of blood. This meant that the dynamics of hormone secretion could be monitored in consecutive samples. It soon became apparent that the release of hormones such as GH (Hunter & Rigal, 1966), ACTH (Berson & Yalow, 1968), and cortisol (Weitzman, Schaumburg & Fishbein, 1966; Hellman, Nakada, Curti *et al.* 1970) into the systemic circulation was in the form of intermittent boluses, and not continuously as had been assumed previously. With further assay refinement, and improvements in sensitivity and precision, it then became clear that LH and FSH were also secreted episodically (monkey: Dierschke, Bhattacharya, Atkinson & Knobil, 1970; bull: Katongole, Naftolin & Short, 1971; human: Midgley & Jaffe, 1971; sheep: Butler, Malven, Willett & Bolt, 1972). Since then, LH pulses have been observed in the sheep and other animals in every reproductive state investigated.

2.1.2. Feedback effects of ovarian steroids on LH secretion

The frequency—and to a lesser extent the amplitude—of LH secretory episodes varies considerably between different reproductive states (Lincoln, Fraser, Lincoln *et al.* 1985). In the luteal phase and during seasonal anoestrus, LH pulses occur once every 3–12 h (Baird, Swanston & Scaramuzzi, 1976; Hauger, Karsch & Foster, 1977; Scaramuzzi & Baird, 1977; Yuthasastrakosol, Palmer & Howland, 1977). LH pulse frequency increases through the follicular phase to reach a rate of one per hour or more prior to the pre-ovulatory LH surge (Hauger *et al.* 1977, Baird, 1978).

This variation has been shown to be due, in part, to the feedback effects of the ovarian steroids, oestradiol and progesterone, and the interactions between them. It became clear that tonic LH secretion was governed by the

negative feedback effects of gonadal steroids when removal of the gonads in sheep (Niswender, Roche, Foster & Midgley, 1968) and rats (Gay & Midgley, 1969) resulted in a massive increase in circulating LH levels. This overall increase in LH release was later shown to be largely due to an increased number of pulses (Butler *et al.* 1972; Diekman & Malven, 1973).

Early work provided circumstantial evidence that progesterone is an important negative feedback steroid. As it was known to be secreted from the corpus luteum at high levels for at least 10 days during the luteal phase of the cycle (Edgar & Ronaldson, 1958; Stabenfeldt, Holt & Ewing, 1969; Moore, Barrett, Brown *et al.* 1969), it seemed likely that it was important in the control of LH secretion. In addition, Hauger *et al.* (1977) demonstrated an inverse correlation between circulating LH and progesterone levels throughout the sheep oestrous cycle, particularly during the follicular phase between the drop from luteal levels of progesterone and the onset of the pre-ovulatory LH surge.

Although it had been shown that single injections of progesterone reduce the frequency of LH pulses in OVX rhesus monkeys (Yamaji, Dierschke, Bhattacharya & Knobil, 1972), the physiological significance of this was not initially appreciated as a pharmacological level of progesterone was required for the effect. Moreover, mean LH levels were unchanged as decreases in frequency were offset by increases in pulse amplitude. However, it was soon demonstrated that a physiological level of circulating progesterone could lower LH levels in the OVX monkey, provided the treatment was sustained by means of a constant release implant and that oestradiol was also present in low concentrations (Karsch, Weick, Hotchkiss *et al.* 1973). This was confirmed later in the sheep. Ovariectomy during the luteal phase caused a prompt increase in LH pulse frequency. Insertion of constant release implants to maintain a mid-luteal phase level of progesterone abolished this post-castration increase in LH pulse frequency (Karsch, Legan, Hauger & Foster, 1977; Goodman & Karsch, 1980). When maintained at a concentration below the luteal phase maximum (e.g., at an early luteal phase level), progesterone alone was shown to be unable to reduce the post-castration rise in LH pulse frequency. However, this low level of progesterone was effective in the presence of a basal concentration of oestradiol (Goodman, Bittman, Foster & Karsch, 1981; Martin, Scaramuzzi & Henstridge, 1983).

Following luteolysis, the levels of progesterone fall in the follicular

phase, leaving oestradiol as the only putative negative feedback hormone, a role which it cannot fulfil during the breeding season (Legan, Karsch & Foster, 1977; Karsch, Legan, Ryan & Foster, 1978). The earliest description of the negative feedback effect of a physiological concentration of oestradiol on the LH pulse-generating mechanism was made in the OVX rhesus monkey (Yamaji, Dierschke, Bhattacharya & Knobil, 1972). Infusion of early- to mid-follicular phase levels of the steroid led to a profound suppression of the hourly discharge of LH pulses. It was subsequently shown that this inhibition of LH secretion could be maintained for months, provided the oestradiol level remained high (Karsch, Dierschke, Weick *et al.* 1973; Karsch, Weick, Hotchkiss *et al.* 1973).

The negative feedback effects of oestradiol on LH secretion were also explored in early studies in the ewe. However, the response pattern in the sheep is more complicated than in the monkey as there are marked seasonal differences in response to oestradiol (Legan, Karsch & Foster, 1977; Goodman, Legan, Ryan *et al.* 1981; Webster & Haresign, 1983; Robinson, Radford & Karsch, 1985). During anoestrus, oestradiol at physiological levels is a potent inhibitor of LH pulse frequency. During the breeding season, however, oestradiol serves primarily to reduce LH pulse amplitude. Physiological levels of the steroid were found to be unable to lower LH pulse frequency in OVX animals at this time (Goodman & Karsch, 1980; Martin, Scaramuzzi & Henstridge, 1983), and in fact enhanced LH pulse frequency in the follicular phase of the oestrous cycle (Karsch, Foster, Bittman & Goodman, 1983). Thus, after luteolysis, oestradiol alone is unable to inhibit the secretion of LH and so plasma LH rises and stimulates oestradiol production by the ovary (Baird, 1978), thus eventually triggering the LH surge and ovulation (Baird & Scaramuzzi, 1976; Karsch, Foster, Legan *et al.* 1979).

It is well established that rising oestrogen levels during the follicular phase, operating via a positive feedback system, are responsible for the LH surge. This was shown by the induction of an LH surge when an oestradiol injection was given to anoestrous ewes (e.g., Goding, Catt, Brown *et al.* 1969; Bolt, Kelley & Hawk, 1971) and to OVX ewes (Scaramuzzi, Tillson, Thorneycroft & Caldwell, 1971). In addition, immunization against circulating oestradiol in normally cycling ewes prevented ovulation (Rawlings, Kennedy & Henricks, 1978; 1979). When endogenous progesterone levels are high, as during the luteal phase, oestradiol is unable to elicit an LH surge (Bolt *et al.* 1971) and in OVX ewes,

exogenous progesterone prevents the normal positive feedback effect of oestrogen (Scaramuzzi *et al.* 1971).

The characteristics of pulsatile LH release during the surge, though difficult to elucidate as frequent sampling is required, have been investigated in several species. Studies in the rat (Gallo, 1981), cow (Rahe, Owens, Fleeger *et al.* 1980), sheep (Karsch, Foster, Bittman & Goodman, 1983) and human (Djahanbakhch, Warner, McNeilly & Baird, 1984) indicate that the LH surge is composed of large pulses of high frequency (up to one pulse per 15–20 min). The pulsatile nature of the pre-ovulatory surge of LH has been investigated in detail, both experimentally and from a theoretical standpoint, in a recent study in the ewe by Martin, Thomas, Terqui & Warner (1987). Their experimental observations showed the surge to be composed of an increase in both LH pulse frequency and amplitude, and that the high frequency of LH pulses was established some time before the onset of the surge proper. Applying a simple mathematical model of pulsatile secretion, the authors concluded their results could only be explained by pulse frequencies and amplitudes well above those normally observed in other physiological situations, and that an increase in pulse frequency was essential for initiation of the surge.

2.1.3. Recognition of the importance of the hypothalamus and the pituitary portal vasculature

There was considerable evidence from early studies, despite the technical limitations of the bioassays available, that the secretion of hormones from the anterior pituitary gland was under the control of releasing factors from the hypothalamus/median eminence.

The role of the nervous system in the regulation of gonadotrophin secretion had been appreciated by Marshall (1936; 1942) as early as 50 years ago and subsequently much research effort was directed towards the question of hypothalamic control of adenohypophysial activity. For instance, hypothalamic stimulation resulted in an increased discharge of LH and ACTH (Harris, 1937; 1948; Haterius & Derbyshire, 1937; Markee, Sawyer & Hollinshead, 1946; de Groot & Harris, 1950). Although many studies addressed the concept of a secretomotor innervation of the anterior pituitary gland, it soon became clear that such an innervation did not exist. With increased appreciation of the significance of the hypophysial portal system—first described by Popa & Fielding (1930; 1933)—attention was directed towards the possibility of a humoral control

mechanism, as originally suggested tentatively by Harris (1937).

A specific action of the pituitary portal blood supply on the activity of the anterior pituitary gland was soon demonstrated by experiments involving pituitary stalk section (e.g. Harris, 1950) and comparison of the function of pituitary grafts under the median eminence with grafts at other sites (Harris & Jacobsohn, 1952; Nikitovitch-Winer & Everitt, 1958; Smith, 1961). These studies showed that anterior pituitary tissue functions apparently normally when vascularized by the hypophysial portal vessels, but not when supplied only by systemic vessels. They provided further evidence in support of the neuroendocrine hypothesis proposed by Green & Harris (1947), whereby the reproductive functions of the anterior pituitary gland are controlled by hypothalamic releasing factors secreted into the pituitary portal blood.

2.1.4. Demonstration of a hypothalamic LH-releasing factor (LRF)

Following on from the above experiments, studies into the existence of a releasing factor for luteinizing hormone were commenced. Crude extracts of median eminence (but not other parts of brain) were shown to cause ovulation in the oestrous rabbit when infused directly into the anterior pituitary gland (Campbell, Feuer & Harris, 1964). In addition, extracts of sheep hypothalamic tissue induced ovulation in rats rendered acyclic by hypothalamic lesions (Schiavi, Jutisz, Sakiz & Guillemin, 1963). McCann and co-workers investigated the effects of i.v. treatment with extracts of stalk/median eminence tissue on LH secretion, as measured by bioassay, in ovariectomized, normal and hypothalamic-lesioned rats and found plasma LH levels to increase rapidly (McCann & Taleisnik, 1961; McCann, 1962; Ramirez & McCann, 1963). These experiments supported the existence of an LRF in the hypothalamus and median eminence.

An LRF of < 5 000 kDa was subsequently demonstrated in the hypothalamus (Ramirez & Sawyer, 1965) and in pituitary stalk blood throughout the cycle (Fink & Harris, 1970). These studies showed an unexpected decrease in secretion at oestrus, with no rise at pro-oestrus, as the neuroendocrine hypothesis predicted. However, the procedures required for sampling of pituitary portal blood required extensive surgery, and there was concern that, as the deep anaesthesia necessary compromised the pre-ovulatory LH surge and blocked ovulation, the hypothalamic secretion of LRF was also inhibited. It was only with the development and application of novel anaesthetics and the use of RIA, that a massive rise in

portal LRF [called LHRH by now] was demonstrated for the first time during the LH surge (Sarkar, Chiappa, Fink & Sherwood, 1976).

2.1.5. Isolation of luteinizing hormone-releasing hormone (LHRH)

These early experiments demonstrated that a low-molecular weight factor was secreted from the hypothalamus into the hypophysial portal blood and acted on the anterior pituitary gland to cause release of LH, and that the concentration of this factor in portal blood increased dramatically at the time of the LH surge.

The factor was subsequently isolated from porcine hypothalamus by Schally, Arimura, Baba *et al.* (1971) and by Amoss, Burgus, Blackwell *et al.* (1971) from ovine hypothalamus. Both these groups concluded that 'LRF' was a nonapeptide containing, on the basis of acid hydrolysis, 1 His, 1 Arg, 1 Ser, 1 Glu, 1 Pro, 2 Gly, 1 Leu, 1 Tyr. However, it was then reported (Matsuo, Baba, Nair *et al.* 1971*b*) that the porcine LRF also contained a residue of tryptophan (Trp) in addition to the other amino acids already observed. The primary structure of LRF from both species was established as pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 with the N-terminal glutamate residue in its cyclized pyroglutamate (pGlu) form and an amidated C-terminal (Matsuo *et al.* 1971*b*; Burgus, Butcher, Amoss *et al.* 1972). Synthesis of such a decapeptide gave the same result as the isolated molecule on sequencing, and analysis of the biological properties of the synthetic peptide showed identical bioactivity with the native form, thus confirming the proposed structure (Matsuo, Arimura, Nair & Schally, 1971*a*; Burgus *et al.* 1972).

Since its isolation and synthesis, LRF has been termed luteinizing hormone-releasing hormone, or LHRH. As it also has the ability to stimulate FSH secretion, it is often referred to as gonadotrophin-releasing hormone (GnRH).

Only one LHRH sequence has been identified in all mammals studied to date. Moreover, salmon LHRH has only two substitutions and chicken LHRH-I differs from mammalian LHRH by only one amino acid: in addition, amphibian LHRH is identical to the mammalian form (see Sherwood, 1986). The high degree of homology between known LHRH structures throughout evolution suggests an important role for this peptide in the regulation of reproductive function. A second LHRH (chicken-II) has also been identified within the chicken brain, which has three amino acid substitutions. Interestingly, chicken-II is considerably

more potent than chicken-I LHRH in releasing both LH and FSH from cultured chicken or rat pituitary cells (see Sherwood, 1986). This supports the possibility that LH and FSH may have separate releasing hormones in avian species.

2.1.6. Evidence for an FSH-releasing factor

As LHRH releases FSH, though to a lesser extent than it does LH (Schally, Redding, Matsuo & Arimura, 1972), and as Schally and co-workers were unable to separate FSH- from LH-releasing activity (Schally, Arimura, Redding *et al.* 1976), these authors concluded that there was only one releasing hormone which controls the release of both gonadotrophins; i.e. the decapeptide LHRH.

This concept, originally put forward by Schally, Arimura, Kastin *et al.* (1971), has attracted much support. However, there is considerable evidence—mostly from McCann's group—that there must be an FSH-RF which is yet to be isolated. For instance, crude extracts of the OVLT contain much more FSH-releasing activity than can be accounted for by the LHRH content, and the slope of the dose-response curve in the bioassay for FSH release is much steeper than that obtained with LHRH (Samson, Snyder, Fawcett & McCann, 1980). Similarly, extracts of the posterior median eminence contain more FSH-releasing activity than can be accounted for by the content of LHRH (Mizunuma, Samson, Lumpkin & McCann, 1983). In a recent study, Lumpkin, Moltz, Yu *et al.* (1987) subjected extracts of sheep pituitary stalk/median eminence tissue to gel filtration and demonstrated FSH-releasing activity in fractions of higher molecular weight than that corresponding to LHRH. These fractions contained no LH-releasing activity measured either by bioassay or by RIA for LHRH. Proof of the existence of a specific FSH-RF, however, must await its definitive isolation and characterization.

Passive immunization against LHRH selectively inhibits LH secretion in the follicular phase ewe, whereas FSH secretion gradually rose in antiserum-treated animals (Fraser & McNeilly, 1983). It could be argued that this supports the hypothesis of a separate FSH-RF. However, as the reduction in LH secretion is likely to reduce ovarian function, the changes in FSH secretion may be attributable to the removal of negative feedback influences of ovarian origin on FSH secretion, such as oestradiol or inhibin. Inhibin, for instance, has been shown to act at a hypothalamic site to suppress FSH release selectively (Lumpkin, Negro-Vilar,

Franchimont & McCann, 1981). In this study, bioactive inhibin was highly purified from rete testis fluid of rams. Injection of the inhibin preparation into the third ventricle of castrate male rats suppressed FSH secretion without affecting plasma LH levels, whereas i.v. administration of purified inhibin was without effect on either gonadotrophin. The response of the pituitary gland to exogenous LHRH was unaffected. This study in itself provides circumstantial evidence for a differential control of FSH and LH release.

Further evidence for an FSH-RF was provided by a recent push-pull perfusion study in the rat. Levine & Duffy (1988) demonstrated that while pulses of LH showed high concordance with LHRH pulses, FSH pulses were not associated with LHRH release in an obvious and consistent manner, less than 45% being temporally associated with LHRH pulses. These authors suggested that either the LHRH/FSH relationships were not easily discernible in these animals, or that an FSH-RF distinct from LHRH may regulate FSH secretion.

Evidence from electrical stimulation studies and lesions supports the concept that specific areas of the rat dorsal AHA are involved in the selective secretion of FSH by the anterior pituitary, whereas the LH-controlling region is thought to be in the POA of the rat (e.g., Chappel & Barraclough, 1976; see also Kalra & Kalra, 1983). Indeed, Kimura & Kawakami (1978) have shown that separate neural pathways control the secretion of LH and FSH in the rat. Whether this of itself provides evidence for a separate FSH-RF is debatable, and the results can be explained by several hypotheses. For instance, it may be that LHRH only stimulates FSH synthesis and that FSH secretion is essentially passive, or alternatively it may be the *temporal pattern* of LHRH secretion that is important.

There is evidence from a recent molecular biological study that the frequency of LHRH stimulation may indeed be of importance in the differential regulation of LH and FSH secretion (Dalkin, Haisenleder, Ortolano *et al.* 1989). A previous study (Leung, Kaynard, Negrini *et al.* 1987) had suggested that ovine LHβ mRNA was increased considerably more than FSHβ mRNA by higher LHRH pulse frequencies. This was investigated further by Dalkin *et al.* (1989), who demonstrated that fast frequency LHRH stimuli (pulses every 8 min) increased α and LHβ mRNA—but not FSHβ mRNA—whereas only FSHβ mRNA was increased by slow frequency LHRH pulses (every 480 min). This therefore

provides a mechanism whereby a single LHRH peptide could selectively control both LH and FSH secretion. Whether it is sufficient to account for the complete differential control of LH and FSH secretion is not known.

2.1.7. Control of LH secretion by LHRH

Clearly, the secretion of LH is pulsatile. But how are these pulses initiated? Several mechanisms have been postulated and subsequently discounted.

It appears that the anterior pitutiary gland has an inherent capacity to secrete at least some of its hormones in a pulsatile fashion. For instance, Shin & Reifel (1981) reported an episodic secretion of prolactin in hypophysectomized male rats in which the anterior pituitary was grafted under the kidney capsule. Pulsatile GH and prolactin release has also been shown from perfused monkey hemipituitaries *in vitro* (Stewart, Clifton, Koerker *et al.* 1985). However, as the pulsatile secretion patterns were much more rapid than those observed *in vivo,* it seems unlikely that the pulsatile release of LH is a result of pituitary oscillations.

A change in pituitary sensitivity also seems unlikely as electrical stimulation of the median eminence at the time of a downward slope of an LH pulse initiated further LH secretion (Malven, 1975). A short-loop negative feedback is also unlikely as continuous infusions of LH failed to inhibit endogenous LH secretion in ovariectomized ewes (Coppings & Malven, 1975).

As described above, it was clear from early studies that; (a) LH secretion is pulsatile and (b) a releasing factor for LH (i.e. LHRH) is secreted into the pituitary portal plasma. Are these two phenomena connected? i.e., are the pulses of LH caused by concomitant pulsatile release of LHRH? This has been investigated in some detail and evidence from a number of different experimental approaches indicates that every LH pulse is precipitated by a corresponding LHRH pulse. Some of these studies are outlined as follows:

Immunoneutralization of endogenous LHRH, by the injection of specific anti-LHRH antibodies, eliminates the pulsatile secretion of LH (ewe: Clarke, Fraser & McNeilly, 1978; McNeilly, Fraser & Baird, 1984; ram: Lincoln & Fraser, 1979; rat: Kawakami & Higuchi, 1979; Snabels & Kelch, 1979; Ellis, Desjardins & Fraser, 1983). In addition, in the female, neutralization with anti-LHRH antibodies inhibits the pre-ovulatory LH surge and the steroid-induced surge of LH (Koch, Chobsieng, Zor *et al.*

1973; Arimura, Debeljuk & Schally, 1974; Blake & Kelch, 1981; Fraser & McNeilly, 1982; 1983). An LH pulse, identical to that observed before immunization, can be induced by the bolus injection of an LHRH agonist that does not cross-react with the anti-LHRH-antibodies in the circulation (Caraty, Martin & Montgomery, 1984).

Some of the best evidence came when pulses of LHRH were measured in the hypophysial portal blood of ovariectomized rhesus monkeys (Carmel, Araki & Ferin, 1976; Neill, Patton, Dailey *et al.* 1977) and rats (Soper & Weick, 1980). These studies had some limitations, as it was not possible to measure LH simultaneously. Since then, however, LHRH has been shown to exhibit tonic pulsatile release, concomitant with plasma LH secretion, into the portal blood of sheep (Clarke & Cummins, 1982) and has been measured coincident with peripheral LH pulses in third ventricular cerebrospinal fluid (CSF) of the rhesus monkey (Van Vugt, Diefenbach, Alston & Ferin, 1985) and push-pull perfusates of the median eminence of sheep (Levine, Pau, Ramirez & Jackson, 1982) and rats (Levine & Ramirez, 1980).

Measurement of portal blood in sheep involves transection of part of the pituitary portal vasculature in the conscious sheep, previously operated upon to give access using a trans-nasal/trans-sphenoidal route. This has permitted samples of blood to be collected over 10- to 15-min periods for up to 12 h or more. Using this technique, an excellent correlation has been observed between LHRH and LH pulses in OVX ewes and at the time of the oestradiol-induced LH surge (Clarke & Cummins, 1982; 1985). This group has applied the portal sampling approach to the analysis of the mechanism of the pre-ovulatory LHRH and LH surges, and those induced by oestradiol benzoate, and also to the investigation of the changes in LHRH secretion that occur throughout the cycle (Clarke, Thomas, Yao & Cummins, 1987). In this study, the levels of LHRH in hypophysial portal blood were observed to surge at the same time as the surge of LH in some ewes but, in other sheep, the LHRH levels did not change, or only increased slightly. These findings could be taken to suggest that the variation in response could be because, as in the monkey, a heightened secretion of LHRH may not be a necessary surge-inducing stimulus in the ewe. However, there is considerable evidence in the ewe that an increased secretion of LHRH and, in particular, an increase in LHRH pulse frequency is required for a normal LH surge.

In the monkey, measurements of LHRH in either pituitary stalk

plasma (Neill, Patton, Dailey *et al.* 1977) or push-pull perfusates of the MBH (Levine, Norman, Gliessman *et al.* 1985; Pau, Gliessman, Hess & Spies, 1988) led to the tentative conclusion that LHRH secretion increased during the oestradiol-induced LH surge. However, as administration of unvarying hourly pulses of LHRH to monkeys with arcuate nucleus lesions was shown to restore the ovulatory LH surge and menstrual cyclicity (Knobil, Plant, Wildt *et al.* 1980), and as a wealth of evidence, both *in vivo* and *in vitro*, demonstrated that the response of the pituitary gland to LHRH was enormously increased by oestrogen (e.g. Reeves, Arimura & Schally, 1971; Drouin, Lagacé & Labrie, 1976), this led to the view that the LH surge depended exclusively on the pituitary gland, and that the hypothalamus provided no more than a 'permissive' signal (e.g. Knobil, 1980). However, it is likely that the hypothalamic lesions did not eliminate the entire endogenous LHRH signal, as two recent anatomical studies in the ewe have concluded. Both Lehman, Robinson, Karsch & Silverman (1986) and Caldani, Batailler, Thiéry & Dubois (1988) have suggested that inputs to the anterobasal OVLT and from the MBH to the ME may remain intact, and some LHRH fibres reaching the ME by the lateral and periventricular pathways may escape lesioning by the knife cuts used in some studies. Moreover, constant LHRH pulse therapy is ineffective when an impervious Teflon barrier is inserted across the pituitary stalk (Norman, Gliessman, Lindstrom *et al.* 1982).

The importance of a change in LHRH pulse frequency in the initiation of the LH surge in the ewe is much less ambiguous. The situation in the sheep appears to be quite different from the received view of the LHRH mechanism operating in the monkey which, according to Knobil and others (e.g. Knobil, 1980), should only be regarded as being 'permissive'.

Studies using two different models and designed to determine the pattern of LHRH secretion required to induce the LH surge in the ewe have clearly indicated that a large 'deterministic' increase in LHRH secretion must occur, and that the primary requirement is for an increase in LHRH pulse frequency. For instance, using a model in which the hypothalamic inputs to the hypophysial portal vessels in the OVX ewe were removed by a surgical hypothalamo-pituitary disconnection (HPD) procedure (Clarke, Cummins & de Kretser, 1983), oestradiol caused only a modest rise in plasma LH levels when hourly pulses of LHRH were given (Clarke & Cummins, 1984). Using another model in which the endogenous secretion of LHRH was suppressed by treatment with

progesterone in the anoestrous ewe, hourly pulses of LHRH were insufficient to restore the positive feedback response of oestradiol, while in progesterone-blocked OVX ewes, half-hourly LHRH pulses plus oestradiol produced an LH surge that was 50% of the peak value obtained in OVX ewes without a progesterone block (Kaynard & Karsch, 1988). Further investigations by the groups of Karsch and Clarke have demonstrated that an abrupt increase in the LHRH input is required to initiate the LH surge (Kaynard, Malpaux, Robinson *et al.* 1988; Clarke, Cummins, Jenkin & Phillips, 1989).

This 'deterministic' view is supported by the recent careful studies of Caraty and co-workers. Using a refinement (Caraty & Locatelli, 1988) of the portal sampling technique described by Clarke & Cummins (1982), Caraty, Locatelli & Martin (1989) showed a clear, consistent and unambiguous increase in the pulsatile secretion of LHRH in response to oestradiol in the ovariectomized ewe. Moenter, Caraty & Karsch (1990) recently applied this technique, refined still further, to an artificial follicular phase model in which oestradiol and progesterone implants were used in ovariectomized ewes in anoestrus and in the breeding season to achieve physiological levels comparable with those of cyclic animals. The essential features of this elegant study were that oestradiol levels were manipulated to mimic those that circulate in the late follicular phase—rather than the more usual pharmacological approach of a single injection—and that this was combined in the same model with an efficient and minimally invasive method of portal blood collection. Regardless of season, the LH surge induced by administration of a follicular phase level of oestradiol was invariably accompanied by a massive and robust surge of LHRH secretion that appeared to be due to an increase in LHRH pulse frequency.

Moenter *et al.* (1990) also showed clearly that the duration of the LHRH surge was extended compared to the length of the LH surge. Moreover, the hypothalamus of the sheep contains 20–80 ng LHRH, whereas each LHRH pulse probably contains less than 100 pg LHRH (Lincoln, 1985). These observations indicate that the LH surge is terminated for reasons other than a lack of LHRH. It is possible that pituitary depletion of LH, or desensitization of the pituitary to LHRH, may contribute to its termination. In this regard, both the pituitary LH content and the number of pituitary LHRH receptors have been shown to decrease at the end of the LH surge in the ewe (Crowder & Nett, 1984; Landefeld, Kepa & Karsch, 1984). As the continuous administration of LHRH to the ewe decreases

the number of pituitary LHRH receptors (Nett, Crowder, Moss & Duello, 1981), the large surge of LHRH may down-regulate the LHRH receptors, thus desensitizing the pituitary gland to LHRH and causing the LH surge to end despite the elevated levels of LHRH.

Alternatively, a specific neuroendocrine factor may be released to suppress LH secretion. A factor with such activity has been extracted from rat hypothalamus and has been shown to inhibit LHRH-stimulated LH release *in vivo* and *in vitro* (Hwan & Freeman, 1987). In addition, the existence of a gonadotrophin surge inhibiting/attenuating factor of ovarian origin that is neither a steroid nor inhibin has been inferred in the monkey and woman—this hypothesis has recently been discussed by Whitehead (1990). Whether such a factor is of physiological relevance in the termination of the LH surge in the sheep is not known at present.

2.1.8. Biosynthesis of LHRH

Seeburg & Adelman (1984) isolated cloned genomic and cDNA sequences coding for LHRH from human placental material. Correct translation of the corresponding mRNA could be predicted to result in the synthesis of a 92 amino acid precursor, having a simple structure comprising three elements. The first part forms a signal peptide, 23 amino acid residues in length, incorporating a hydrophobic centre region. This is followed by the mature LHRH decapeptide. Cleavage at the N-terminal site exposes the N-terminal glutamate residue which is cyclized to pyroglutamate. The native hormone is flanked at the C-terminus with a tripeptide -Gly-Lys-Arg- sequence. This provides proteolytic cleavage sites and donates the amino group for amidation of the C-terminal glycine residue. The processing site is followed by the remaining 56 amino acids of the precursor. After post-translational processing, the precursor gives rise to two peptides: LHRH and the 56 amino acid flanking peptide which Seeburg & Adelman (1984) termed GnRH-associated peptide (GAP).

The presence in rat brain of an identical prohormone for LHRH was confirmed by immunocytochemical co-localization of LHRH and GAP in cell bodies in the POA and septal complex, and in terminal fields in the OVLT and ME (Phillips, Nikolics, Branton & Seeburg, 1985). In addition, cDNA coding for the same precursor has been characterized from human and rat hypothalamus (Adelman, Mason, Hayflick & Seeburg, 1986).

The co-secretion of LHRH and GAP into pituitary portal blood has been demonstrated by Clarke, Cummins, Karsch *et al.* (1987), though as the

secretion ratio was not 1: 1, this may reflect a further processing to smaller peptides. This co-release is of considerable interest, as GAP has been shown to be a highly potent prolactin inhibiting factor *in vitro* (Nikolics, Mason, Szönyi *et al.* 1985), and passive immunization of rabbits against GAP-related fragments resulted in highly elevated plasma prolactin levels (Nikolics & Seeburg, 1986). The existence of a prolactin inhibiting sequence within the LHRH precursor may explain partially the inverse relationship between LH and prolactin secretion that occurs during lactation, seasonal breeding and infertility.

Reproductive functions in all mammalian species investigated to date are uniquely dependent on a single gene which encodes the LHRH-GAP precursor (see Seeburg, Mason, Stewart & Nikolics, 1987). This group used a mouse strain with a mutation that results in a hypogonadal (*hpg*) phenotype characterized by undetectable levels of hypothalamic LHRH and of circulating gonadotrophins (Cattanach, Iddon, Charlton *et al.* 1977). As LHRH administration elevated circulating gonadotrophin levels, and as foetal pre-optic tissue grafted into the third ventricle of *hpg* mice resulted in the synthesis and secretion of LHRH with a partial restoration of gonadtrophin secretion, an isolated LHRH deficiency was proposed to be the cause of the hypogonadism (Krieger, Perlow, Gibson *et al.* 1982; Charlton, Halpin, Iddon *et al.* 1983; Gibson, Krieger, Charlton *et al.* 1984).

Analysis of the LHRH gene in *hpg* mice revealed a large deletion which removed the third and fourth exons encoding the 45 C-terminal residues of GAP (Mason, Hayflick, Zoeller *et al.* 1986*a*). Interestingly, the second exon encoding LHRH and the first 11 residues of GAP was left intact, as was the first exon and the 5′ DNA regions thought to control the tissue-specific expression of the gene. Although *in situ* hybridization of hypothalami from *hpg* mice revealed mRNA containing the coding sequence for LHRH in POA neurones, the mutant mRNA does not generate any functional LHRH or GAP, as shown by immunocytochemistry (Mason *et al.* 1986*a*). The architecture and neuronal circuitry of the hypothalamus appears to be intact in *hpg* mice, and thus a non-functional LHRH gene seems to be the sole cause of the phenotype.

Mason, Pitts, Nikolics *et al.* (1986*b*) restored the reproductive capacity of the *hpg* mouse by creating a transgenic construct incorporating the normal mouse LHRH-GAP gene. This was introduced into the mutant genome through a series of crosses between heterozygous *hpg* mice and transgenic wild-types. The transgenic homozygous *hpg* offspring all showed a

complete phenotypic reversal of the *hpg* mutation, with normal gonadal development, ovulatory cycles and fertility. The presence of LHRH, GAP and the corresponding mRNA sequences was demonstrated by immunocytochemistry and *in situ* hybridization in the hypothalamus. These experiments demonstrate clearly that reproductive functions in mammalian species are critically dependent on a single LHRH-GAP gene. In the human, this gene has been located on the short arm of chromosome 8 (Yang-Feng, Seeburg & Francke, 1985).

2.1.9. Anatomical localization of LHRH in brain

Neurones immunoreactive for LHRH have been located scattered throughout the mediobasal hypothalamus, in the anterior hypothalamic and preoptic areas, and also in extrahypothalamic sites such as the septal complex and other parts of the forebrain (Jennes & Stumpf, 1980; Silverman, Antunes, Abrams *et al.* 1982; Witkin, Paden & Silverman, 1982; Hoffman, 1983; Lehman, Robinson, Karsch & Silverman, 1986; Caldani, Batailler, Thiéry & Dubois, 1988). Unlike the posterior pituitary neurones, LHRH cells are not tightly organized within clearly defined hypothalamic nuclei and nowhere are the neurones very numerous: there are only about 2 500 in the entire sheep brain (Caldani *et al.* 1988).

There appears to be a species difference in the distribution pattern of LHRH-ir in the brain. LHRH neurones occur in relatively high density in the MBH of the monkey (Silverman, Antunes, Abrams *et al.* 1982), whereas in the sheep (Glass, Mastran & Nett, 1986; Lehman *et al.* 1986; Caldani *et al.* 1988) and also in the rat (e.g., see Silverman, Krey & Zimmerman, 1979) they are more concentrated in the pre-optic and anterior hypothalamic areas. Although Lehman *et al.* (1986) found only 1–2% of LHRH perikarya in the MBH, Caldani *et al.* (1988) found 15% of the total number of LHRH cells in nuclei in this area. This rigorous study, and that of Advis, Kuljis & Dey (1985), showed the great majority of LHRH-ir cells in the sheep to be located in the preoptico-hypothalamic area, mainly in the pre-optic area surrounding the OVLT.

The two major projections of neurones originating in the entire preoptico-hypothalamic area are the OVLT and the ME (Lehman *et al.* 1986; Caldani *et al.* 1988). Caldani and co-workers concluded that there were four major pathways for LHRH fibres. LHRH cells located in the more anterior structures of the POA send fibres to the anterobasal part of the OVLT. Three other pathways were observed to terminate in the ME: a

mediobasal pathway from the POA to the anterior ME; a laterobasal path from the lateral preoptic and lateral hypothalamic areas, joined also by fibres from more dorsal areas around the anterior commisure, to the dorsolateral edges of the central ME; and a periventricular pathway from the POA, along the lateral walls of the supraoptic recess and the third ventricle, to the infundibular recess. While some of these latter neurones were observed to terminate in the ependyma of the ventricle, the majority joined the laterobasal pathway to terminate in the dorsolateral ME. The physiological significance of the different pathways, is currently unknown.

There is morphological evidence from this study (Caldani *et al.* 1988) and from ultrastructural analysis (Jennes, Stumpf & Sheedy, 1985), that suggests the existence of sub-populations of LHRH neurones in the anterior hypothalamic regions. The function of these different LHRH neurone sub-types is likewise not known at present.

Pulsatile LHRH secretion is powerfully regulated by the feedback of gonadal steroids and by photoperiod. It is clear that ovarian steroids exert a major part of their effects on LH secretion in the ewe largely through a hypothalamic action on LHRH secretion. However, the results of studies utilizing a combination of autoradiographic methods to identify neurones that sequester oestradiol and immunocytochemical techniques to localize cells containing LHRH, suggest that target cells for oestradiol in the hypothalamus may be different from LHRH neurones, though the distributions of these neurones overlap (Shivers, Harlan, Morrell & Pfaff, 1983). Neurones nearby, however, have been observed to accumulate abelled oestradiol and many appear also to contain γ-aminobutyric acid GABA; Flügge, Oertel & Wuttke, 1986). Therefore, it is possible that oestradiol could act through a GABAergic system to inhibit LHRH secretion (Lamberts, Vijayan, Graf *et al.* 1983). This does not necessarily exclude LHRH cells as targets for oestradiol as steroid hormones may act directly on neurones through pathways other than classical receptor-mediated genomic mechanisms (McEwen, Biegon, Davis *et al.* 1982).

Lehman, Robinson, Karsch & Silverman (1986) have suggested that here may also be differences in the morphological characteristics of LHRH neurones between the breeding and non-breeding seasons. Although the mall number of animals in this study means that no firm conclusions an be made, the total dendritic length of LHRH neurones in luteal phase ewes appears to be much shorter than those neurones in anoestrous ewes. Moreover, these authors have also shown that the density of synaptic

inputs to LHRH neurones in the POA is significantly greater in the mid-luteal phase of the oestrous cycle than in anoestrus (Karsch, Lehman & Silverman, 1987). In addition, Glass, Mastran & Nett (1986) investigated the effects of oestradiol and progesterone on LHRH-ir neuronal systems in anoestrous ewes, and found the number of LHRH cell bodies that were multipolar in animals treated with oestradiol was twice the number in control ewes, while treatment with progesterone caused a significant decrease in POA LHRH content.

In an ultrastructural analysis of LHRH neurones in anoestrous ewes, Lehman, Karsch, Robinson & Silverman (1988) demonstrated that LHRH axons and terminals in the ME were almost entirely surrounded by glial processes, as were LHRH cell bodies in the POA. The authors suggested that the encapsulation of LHRH terminals by glial cells could modulate the activity [stimulus-secretion coupling] of the LHRH neurones, and that the degree of glial ensheathment may change with season. This is especially interesting as glial elements have been shown to play an important role in regulating the activity of magnocellular neurosecretory cells in the rat (Hatton, Perlmutter, Salm & Tweedle, 1984; Theodosis & Poulain, 1984). In late pregnancy and lactation for instance, the magnocellular neurones enlarge, the astrocyte processes retract from between the neurones, and there is a concomitant increase in the number of synaptic inputs to the magnocellular neurones. Glial elements also appear to regulate the access of magnocellular neurosecretory axons to blood vessels in the posterior pituitary gland (Tweedle & Hatton, 1987). More dynamic changes of this type could contribute to the modulation of pulsatile LHRH release. Additionally, it is clear from evidence obtained in the rat that LHRH-containing synapses are located on dendrites and perikarya of LHRH neurones (Leranth, Segura, Palkovits *et al.* 1985; Pelletier, 1987); this observation could provide a further anatomical substrate for the interaction between LHRH neurones and the orchestration of pulsatile activity (Dyer & Robinson, 1989).

Light microscopic studies have suggested several candidates for the neurochemical identity of afferents to LHRH cells in the POA, including the catecholamines, dopamine and noradrenaline (Hoffman, Wray & Goldstein, 1982; Jennes, Beckman, Stumpf & Grzanna, 1982) and several peptides, including neurotensin and substance P (Hoffman, 1985). In addition, Kiss & Halász (1985) have shown the termination of serotoninergic neurones on LHRH cells in the POA of the rat. Of these

neurotransmitters, dopamine and noradrenaline have been implicated in the control of seasonal breeding in the ewe (Meyer & Goodman, 1985; 1986). Close contacts between LHRH neurones and those staining for tyrosine hydroxylase and dopamine β-hydroxylase (the synthetic enzymes for dopamine and noradrenaline) have also been observed (Lehman, Karsch & Silverman, 1988).

As discussed later in this chapter, there is also much evidence from studies of the rat—including immunohistochemical data (e.g., see Guy, Li & Pelletier, 1988)—that the recently discovered hypothalamic peptide, neuropeptide Y, may be of importance in modulating the activity of the hypothalamic-ovarian axis and also the hypothalamic-adrenal axis.

2.1.10. Electrophysiological correlates of LHRH secretion

LHRH secretion is assumed to be evoked by action potential-induced depolarization of LHRH nerve terminals and the consequent exocytosis of secretory granules containing LHRH. As it is clear from analysis of cephalic arterio-venous differences in concentration that LH pulses are secreted over a very short (i.e., 2–6 min) time frame (Rasmussen & Malven, 1982), a pulse of secretion is probably a result of a brief but relatively synchronized burst of activity occurring throughout a population of LHRH nerve endings (Lincoln, Fraser, Lincoln *et al.* 1985).

There is much evidence that electrical stimulation of the POA and the ME readily evokes ovulation and release of LHRH and LH (Kawakami, Terasawa & Ibuki, 1970; Fink & Jamieson, 1976; Dyer, Mansfield & Yates, 1980) and thus much research effort has been directed at determination of the electrical events associated with pulsatile LHRH secretion. However, this has proved to be difficult to achieve for several reasons. Firstly, the rat—though much used for electrophysiological studies, particularly with respect to the posterior pituitary—is just too small to monitor pulsatile LH secretion reliably under conditions permitting effective electrical recording. In addition, many of the anaesthetics used to facilitate the recording of electrical activity adversely affect pulsatile LH secretion. Secondly, as LHRH neurones are so relatively sparse, and as they are not located in discrete nuclei but scattered throughout the pre-optic area and anterior hypothalamus, detection for electrophysiological recording is a major problem.

To overcome these difficulties, recordings of multi-unit activity (MUA) have been made which integrate the electrical activity of the surrounding

neural tissue, recording in an additive fashion the spike activity of many cells, axons and nerve endings. Although early studies by Kawakami *et al.* (1970) had shown a characteristic increase in MUA activity in the pre-optic/anterior hypothalamic areas of the rat on the afternoon of pro-oestrus, it was not until more recently that explosive and transient increases in hypothalamic electrical activity have been correlated with pulsatile LH secretion in the plasma of the rat (Kawakami, Uemura & Hayashi, 1982). Thiéry & Pelletier (1981) recorded MUA of neurones in the antero-medial hypothalamic areas adjacent to the anterior median eminence in the sheep and described an increase in bursting at the initiation of an LH pulse. However, not every increase in MUA was associated with a secretory episode of LH. In a further study in the ewe, Martin & Thiéry (1987) also observed a second type of cellular electrical activity in addition to that which they had observed previously in the median eminence/medial retrochiasmatic area. This second type of activity was observed in more lateral areas and decreased before the onset of LH pulses, increasing again once secretion had begun. This may represent the activity of an inhibitory input to the LHRH neurones.

Recordings of great clarity have been obtained by Knobil's group in the rhesus monkey by implanting electrode arrays in the arcuate nucleus (Wilson, Kesner, Kaufman *et al.* 1984). In this study, the selection of an electrode picking up a signal with a one-to-one relationship with the release of LH pulses showed that each LH pulse was accompanied by an abrupt rise in multi-unit activity of 1–2 min duration, followed by a plateau phase of lesser, but still increased, activity. The duration of this plateau phase is reduced by the administration of oestradiol to OVX animals (Kesner, Wilson, Kaufman *et al.* 1987).

The results from the monkey, however, should be interpreted with caution. The structure in the sheep brain equivalent to the monkey arcuate nucleus is more commonly known as the infundibular nucleus, and contains relatively much fewer LHRH cell bodies (Lehman *et al.* 1986; Caldani *et al.* 1988). In the monkey, every increase in MUA was associated with an LH pulse. However, recording from the medial POA of the sheep, some increases in MUA were not associated with LH secretory episodes and, recording from certain more lateral areas, LH pulses were correlated with a decrease in MUA. It is possible, therefore, that in the experiments in the monkey, recordings have been made from an input to the LHRH neurones.

The most likely explanation for the pattern of LHRH secretion throughout the cycle as it relates to the pattern of electrophysiological activity, is that it represents an interaction between or summation of two or more periodic stimulatory and inhibitory signals (Martin & Thiéry, 1987). The overall pattern in this composite signal throughout the reproductive cycle may depend on the relative degree to which one of these signals dominates the other(s).

2.2. Hypothalamic control of the pituitary-adrenal axis

Under conditions of physical and environmental stress, the body responds with the activation of the hypothalamo-pituitary-adrenocortical axis. Glucocorticoids—cortisol in sheep and man, corticosterone in rat—are released in pulses from the adrenal cortex in response to pulses of adrenocorticotrophic hormone (corticotrophin; ACTH) secreted from the pituitary corticotrophs.

As described above for the hypothalamo-pituitary-ovarian axis, the neuroendocrine hypothesis of Harris led to a search for a hypothalamic releasing factor for ACTH. However, the identity of this putative neuropeptide has proved elusive and it has become evident that the view of a single corticotrophin-releasing factor (CRF) was too simple and that there are several neuropeptides involved in the control of ACTH release.

The principal secretagogue for ACTH in the rat is corticotrophin-releasing hormone (CRH), first isolated by Wylie Vale and co-workers from ovine hypothalamus (Vale, Spiess, Rivier & Rivier, 1981; Vale, Rivier, Brown *et al.* 1983). However, it is clear that arginine vasopressin (AVP) also has corticotrophin-releasing properties and that CRH and AVP act synergistically in the control of ACTH release (Gillies, Linton & Lowry, 1982; Rivier & Vale, 1983). In addition, substances such as oxytocin, angiotensin II, adrenaline, noradrenaline and opioid peptides have all been implicated as ACTH secretagogues (reviews: Antoni, 1986; Gaillard & Al-Damluji, 1987). The terminology in this field is complicated by the fact that the specific hormone, CRH, is often referred to as 'CRF'. However, this is not very helpful as CRH, AVP and angiotensin II are all corticotrophin-releasing factors, i.e. they are all CRFs. As discussed above, the 'CRF complex' consists of many CRFs, including CRH. Therefore, to avoid confusion CRH will be referred to as such throughout the thesis,

and the general term CRF reserved for factor(s) with corticotrophin-releasing properties.

Although AVP is only a weak secretagogue for ACTH in the rat, much evidence suggests that it possesses far greater ACTH-releasing activity in other species, particularly the sheep. The relative potency of CRH and AVP as ACTH secretagogues in the sheep is controversial, however. Pradier, Davicco, Safwate *et al.* (1986) reported a greater release of ACTH after AVP than after CRH, following i.v. administration of the same weight of peptide, whereas Brooks & Challis (1989) found CRH to be the more potent of the two on an equimolar basis. Redekopp, Livesey, Toth & Donald (1985) observed similar peak increases in ACTH, but a more prolonged effect after CRH. Interestingly, while Brooks & Challis (1989) demonstrated that CRH produced a greater peak ACTH response, they found a greater peak cortisol response after AVP. The total amount of cortisol released in the 240 min after injection, however, was no different between the CRH- and AVP-treated animals. These differences in peak cortisol responses raise the possibility that AVP may have a direct influence on cortisol release from the adrenal gland, or alternatively that different molecular forms of ACTH with different biological activities may be released after CRH or AVP (Pradier, Dalle, Tournaire & Delost, 1988). Brooks & Challis (1989) demonstrated clearly that CRH and AVP act synergistically in the sheep—as in the rat and human (see Antoni, 1986)—to release ACTH.

CRH is synthesized in cell bodies located in the parvocellular subdivisions of the PVN, and these areas project extensively to the external zone of the ME (Swanson, Sawchenko, Rivier & Vale, 1983). In the intact rat, AVP is co-localized in 1–2% of the CRH-stained neurones in the parvocellular PVN, though this proportion is greatly increased by adrenalectomy (Sawchenko, Swanson & Vale, 1984). Both CRH and AVP are present in portal blood in concentrations capable of stimulating ACTH release *in vitro* (Gibbs & Vale, 1982; Plotsky, Bruhn & Vale, 1984), and have been shown to increase sharply in the hypophysial portal blood of cat, rat and sheep in response to acute haemorrhage (Carlson & Gann, 1984; Plotsky, Bruhn & Vale, 1985*a*; Caraty, Grino, Locatelli & Oliver, 1988). Basal secretion of both CRH and AVP from the hypothalamus into the pituitary portal system of the sheep is pulsatile (Caraty *et al.* 1988; Engler, Fham, Fullerton *et al.* 1989) and is increased in response to insulin-induced hypoglycaemia—as is portal AVP secretion in the rat (Plotsky,

Bruhn & Vale (1985*b*)—and to an audiovisual stress such as a barking dog (Engler *et al.* 1989).

In summary, the hypothalamo-pituitary-adrenal axis is controlled by complex regulatory mechanisms. Stress increases the secretion of CRH, AVP and possibly other CRFs from the hypothalamus into the pituitary portal blood which then act either alone or in synergy to control the release of the stress hormone ACTH from the anterior pituitary gland. ACTH secretion is also under the inhibitory influence of the adrenal glucocorticoids which act at the hypothalamic and/or pituitary level.

2.3. Neuropeptide Y

In the last decade there has been a vast increase in the literature relating to the recently discovered hypothalamic peptide, neuropeptide Y. This introductory section can only aim to provide a selective overview of the literature to set the studies reported in the main body of the thesis in context. For additional information, see the comprehensive review by McDonald (1988) and the series of monographs edited by Mutt, Fuxe, Hökfelt & Lundberg (1989).

2.3.1. Historical aspects

The discovery of neuropeptide Y began with the isolation of gastro-intestinal peptides from the porcine small intestine by Viktor Mutt's group at the Karolinska Institute in Stockholm. By the mid-1970s, these workers had isolated several peptide hormones—including secretin, cholecystokinin (CCK), and vasoactive intestinal polypeptide (VIP)—which all had C-terminal α-carboxamide structures rather than free carboxyl groups (see Mutt, 1989). Although the nonapeptides oxytocin and vasopressin share this C-terminal amide, as does LHRH, the structure is uncommon amongst peptides. It therefore seemed possible to make use of their C-terminal amide groups to identify these peptides in tissue extracts. It also seemed possible that a search for this characteristic structure in extracted tissue might lead to the discovery of as yet unknown hormonal peptides (Mutt, 1976).

Tatemoto and Mutt therefore developed a new chemical method to detect C-terminal amide groups. This involved endopeptidase cleavage of the C-terminal amino acid α-amides, conversion of these into their

fluorescent dansyl derivatives and subsequent identification using two-dimensional thin-layer chromatography. The method was successfully used to monitor the chromatographic purification of secretin (Tatemoto & Mutt, 1978). Applying this technique to side-fractions of the purification of secretin, it was clear that these fractions contained as yet unknown peptides with the C-terminal α-amide structure. Two such peptides were isolated in this fashion, one with a C-terminal isoleucine amide, and the other with a C-terminal tyrosine amide (Tatemoto & Mutt, 1980).

The first was found to be a 27 amino acid peptide with an N-terminal histidine and was named peptide histidine-isoleucine (PHI) on the basis of its structure. The amino acid sequence was subsequently determined and found to have a high degree of homology with VIP and secretin (Tatemoto & Mutt, 1981).

At first, it seemed probable that the other peptide was pancreatic polypeptide (PP) as this had already been isolated and found to have a C-terminal tyrosine amide (Lin & Chance, 1974; Chance, Johnson, Hoffman & Lin, 1979). However, this turned out not to be the case. Although it was found (like PP) to be a 36 amino acid peptide, it had an N-terminal tyrosine, unlike the alanine of porcine PP. It was therefore named peptide tyrosine-tyrosine (PYY). The complete sequence of pPYY was determined by Tatemoto (1982*a*) and found to have considerable homology with pPP.

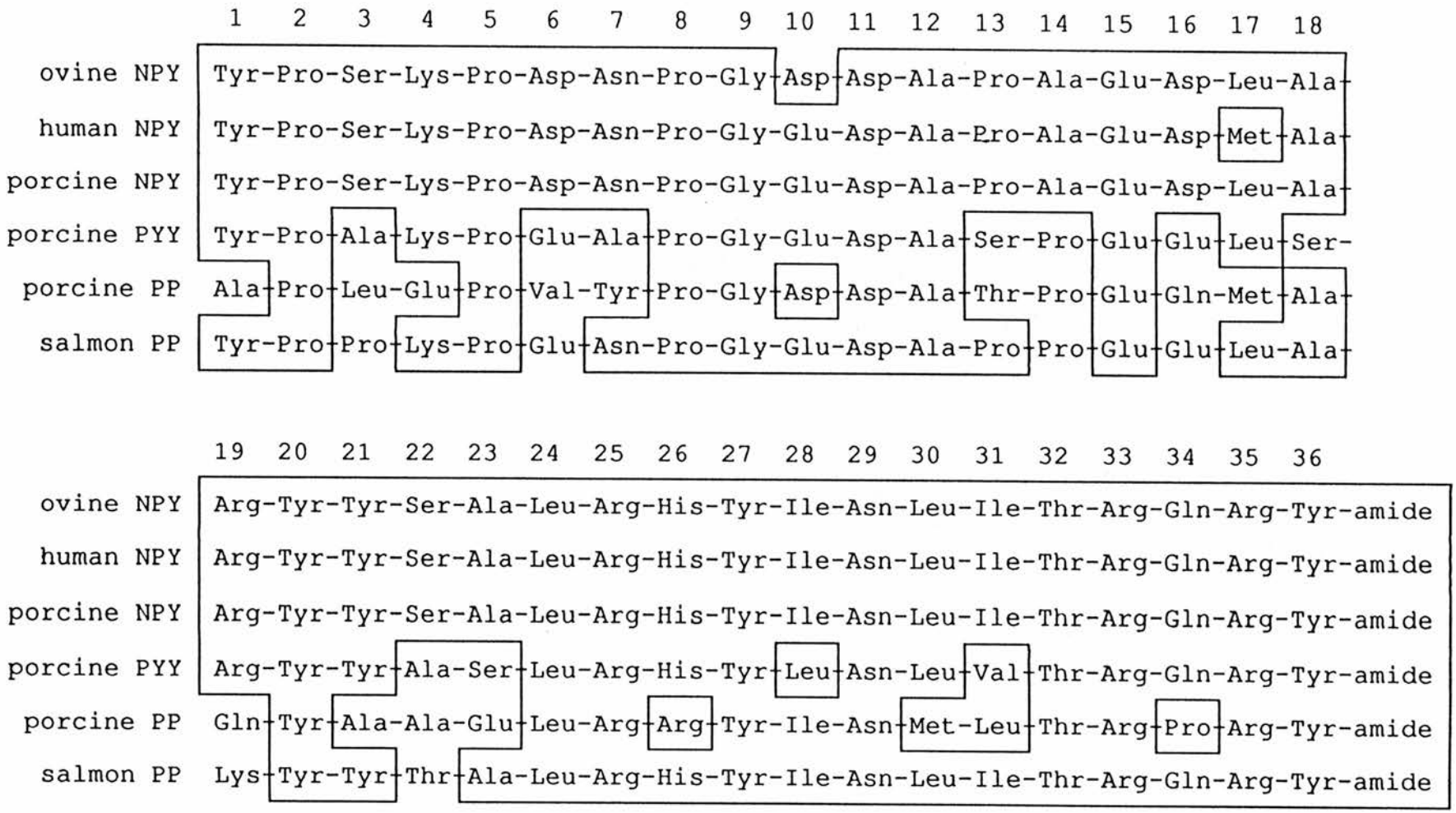
2.3.2. Isolation and structure

It has been known for some years that many peptide hormones exist in the brain and periphery—particularly the intestine—in identical forms; e.g., angiotensin II, substance P, VIP and members of the CCK/gastrin family (see Hökfelt, Johansson, Ljungdahl *et al.* 1980). Tatemoto and Mutt therefore applied their new technique to extracts of porcine brain and found peptides with C-terminal isoleucine amide and tyrosine amide. These were expected to be PHI and PYY. However, although the first peptide amide did indeed turn out to be PHI (Tatemoto, Carlquist, McDonald & Mutt, 1983), instead of PYY they found a related 36 amino acid peptide, also with an N-terminal tyrosine (Tatemoto, Carlquist & Mutt, 1982). This was named neuropeptide tyrosine (neuropeptide Y; NPY) to distinguish it from PYY. The complete amino acid sequence was elucidated by Tatemoto (1982*b*) and found to have 69% homology with pPYY and 50% with pPP (see Figure 2.1).

NPY has since been isolated from other mammals and shows

Figure 2.1. Comparison of the primary structure of porcine neuropeptide Y with ovine and human NPYs. Compare also the structures of porcine peptide YY, porcine pancreatic polypeptide and salmon PP. Identities with pNPY are enclosed by the box. Ovine NPY only differs from pNPY or from hNPY by one [conservative] amino acid substitution. Bovine NPY (not shown) is identical in structure to pNPY, whereas rat, guinea pig and rabbit NPYs (not shown) are all identical to hNPY. Note the close sequence homologies of this family of peptides throughout evolution: the N-terminal poly-proline helix is particularly highly conserved. See text for further details.

Sources of the data shown are as follows: oNPY: Sillard *et al.* (1989); hNPY: Corder *et al.* (1984), Minth *et al.* (1984); pNPY: Tatemoto (1982*b*); pPYY: Tatemoto (1982*a*); pPP: Lin & Chance (1974), Chance *et al.* (1979); salmon PP: Kimmel *et al.* (1986).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
ovine NPY Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Asp-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-
human NPY Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-
porcine NPY Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-
porcine PYY Tyr-Pro-Ala-Lys-Pro-Glu-Ala-Pro-Gly-Glu-Asp-Ala-Ser-Pro-Glu-Glu-Leu-Ser-
porcine PP Ala-Pro-Leu-Glu-Pro-Val-Tyr-Pro-Gly-Asp-Asp-Ala-Thr-Pro-Glu-Gln-Met-Ala-
salmon PP Tyr-Pro-Pro-Lys-Pro-Glu-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Pro-Glu-Glu-Leu-Ala-
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
ovine NPY Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-amide
human NPY Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-amide
porcine NPY Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-amide
porcine PYY Arg-Tyr-Tyr-Ala-Ser-Leu-Arg-His-Tyr-Leu-Asn-Leu-Val-Thr-Arg-Gln-Arg-Tyr-amide
porcine PP Gln-Tyr-Ala-Ala-Glu-Leu-Arg-Arg-Tyr-Ile-Asn-Met-Leu-Thr-Arg-Pro-Arg-Tyr-amide
salmon PP Lys-Tyr-Tyr-Thr-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-amide

remarkable conservation between species. The amino acid sequences of human (Corder, Emson & Lowry, 1984; Minth, Bloom, Polak & Dixon, 1984), rat (Corder, Gaillard & Böhlen, 1988), rabbit and guinea pig (O'Hare, Tenmoku, Aakerlund *et al.* 1988) NPY are all identical and differ from pNPY only at position 17, where the porcine structure has a leucine residue and the others a methionine (see Figure 2.1). Recently, NPY has been isolated from the brain of the cow and the sheep. Whereas the amino acid sequence of bovine NPY is identical to that of pNPY (Tatemoto, 1989), ovine NPY differs from pNPY by one amino acid at position 10. The porcine structure has a glutamate residue at this position, whereas the corresponding amino acid in oNPY is aspartate (Sillard, Agerberth, Mutt & Jörnvall, 1989). Moreover, NPY has strong structural similarities to fish PP. Whilst pNPY has 50% homology with pPP, it enjoys 83% with salmon PP isolated from the endocrine pancreas of the Pacific salmon, having 30 out of 36 amino acids in common with this peptide (Kimmel, Plisetskaya, Pollock *et al.* 1986) (see Figure 2.1).

The degree of sequence homology between these various peptides and the inability of investigators to isolate PP from the brain led to speculation that the PP-ir previously observed in the brain was actually due to cross-reactivity of these antisera with endogenous NPY (see McDonald, 1988). This was shown when pre-absorption of PP antisera with NPY prevented the appearance of PP-ir in neural tissue (Lundberg, Terenius, Hökfelt & Tatemoto, 1984). In addition, further characterization by RIA of PP-ir from chromatographed rat brain extracts showed that this immunoreactivity coeluted with synthetic NPY (DiMaggio, Chronwall, Buchanan & O'Donohue, 1985). These and other findings have led to a concensus view hat accounts of PP-ir in the brain are in fact largely due to cross-reactivity vith the endogenous peptide NPY (McDonald, 1988).

The inter-species homologies between the different NPYs and between ther members of the PP family suggest that these peptides have been ighly conserved throughout evolutionary history. Moreover, the nembers of this peptide superfamily are characterized by a common, listinct tertiary structural feature, the 'PP-fold' (Glover, Barlow, Pitts *et al.* 985). This consists of two anti-parallel helices—an N-terminal poly-roline helix and a long hydrophilic α-helix, connected by a β-turn, erminating in a mobile polar C-terminal hexapeptide amide—held ogether by tightly packed interdigitating hydrophobic side chains Schwartz, Fuhlendorff, Langeland *et al.* 1989). Overall, the N- and C-

termini are brought into close spatial proximity.

Certain structural requirements for the biological and pharmacological actions of NPY are explained by the tertiary structure. At least two receptor populations—Y_1 and Y_2 of differing affinity and specificity—have been identified on the basis of NPY fragment bioactivity (Wahlestedt, Yanaihara & Håkanson, 1986) and radioligand binding (Schwartz *et al.* 1989). Based on results from bioassays, Wahlestedt *et al.* (1986) proposed that two distinct receptors were responsible for the pre- and post-synaptic effects of the peptide respectively. In the test systems they used, the post-junctional effect (vasoconstriction) could only be obtained from the whole NPY molecule, whereas the $NPY_{(13-36)}$ amide fragment was sufficient to elicit the pre-junctional effect (inhibition of noradrenaline release). However, this classification has not gained wide general acceptance as the long C-terminal fragment has been shown to give significant post-junctional effects in other test systems (see Schwartz *et al.* 1989). A classification has therefore been proposed based on differing affinities and specificities for the whole molecule and for the C-terminal fragment (residues 13–36).

Both receptors require the presence of the amide residue on the C-terminal tyrosine. The Y_1 receptors have been identified mainly at post-synaptic sites in the sympathetic nervous system, and require the whole NPY molecule for activation. This sub-type of receptors binds NPY with a dissociation constant (K_d) in the lower nanomolar range, but does not bind long C-terminal fragments such as $NPY_{(13-36)}$ amide (K_d approximately nicromolar) (Schwartz *et al.* 1989). This is in keeping with a compact ertiary structure in which the N- and C-terminal regions are in close oroximity. The Y_2 receptor, which is predominant in the CNS, was riginally defined as a pre-synaptic receptor (Wahlestedt *et al.* 1986), but it s now clear that it is also found post-junctionally (see Schwartz *et al.* 989). It binds NPY with a much lower K_d than the Y_1 receptor, i.e. in the ub-nanomolar range. The minimum active fragment length for the Y_2 eceptor is $NPY_{(13-36)}$ amide, the K_d for the binding of this fragment being pproximately nanomolar. This fragment comprises the C-terminal polar rm and the α-helix. Activity at this site is thus dependent on the ntegrity of the α-helix.

On the basis of the receptor activities and distribution of the various PP-fold' peptides, Schwartz *et al.* (1989) have proposed an evolutionary cheme for the development of the PP peptide family from a common ncestral PP-fold peptide into two lineages, the NPY line and the PP line.

They suggested that in each of these lines there is a central neuropeptide and a peripheral peptide hormone. In the NPY line these correspond to NPY and PYY; in the PP line they proposed PP as the peripheral hormone, paired with an as yet unidentified central neuropeptide. Interestingly, in a personal communication from Tatemoto, Schwartz *et al.* (1989) reported structural evidence for the presence in porcine brain of a new PP-fold peptide with an alanine residue in position one.

2.3.3. Biosynthesis

Minth *et al.* (1984) were the first to identify mRNA and synthesize cDNA responsible for the structure of NPY. In-vitro translation of the recombinant RNA demonstrated that the tumour contained a mRNA encoding a 10.8 kDa protein, which could be immunoprecipitated with antiserum raised against NPY. They isolated RNA from a human pheochromacytoma and prepared a cDNA library from this, identifying the NPY cDNA using a mixture of hybridization probes based on the known amino acid sequence. Positive clones were isolated and their nucleotide sequences determined. The coding sequence consisted of 291 bases, thus suggesting a precursor to NPY that was 97 amino acids in length.

The deduced amino acid sequence of the precursor (preproNPY) revealed two potential sites of proteolytic processing which Minth *et al.* (1984) postulated would generate three peptides: a signal peptide of 28 amino acid residues, hNPY (36 amino acids), and a 30 amino acid residue C-terminal flanking peptide. The mature 36 amino acid hNPY sequence is flanked at the C-terminal tyrosine by a -Gly-Lys-Arg- tripeptide structure which is converted to the amidated tyrosine residue during post-translational processing.

Using the cDNA as a probe, Minth, Andrews & Dixon (1986) isolated, characterized and sequenced the cloned hNPY gene. They found the transcription unit to span ~8 kilobase pairs and to consist of 4 exons. The first exon contains only non-translated DNA. The second codes for the signal peptide and mature NPY. This exon begins with the initiator methionine for preproNPY and extends to the arginine residue which precedes the C-terminal tyrosine amide of mature NPY. The third exon contains the coding region for the 4 amino acids that are processed to the tyrosine amide structure, plus the first 23 amino acid residues of the C-terminal flanking peptide of NPY (CPON). The fourth codes for the

remainder of the CPON and also the 3′ non-translated region. The hNPY and hPP genes have been located on human chromosomes 7 and 17, respectively (Takeuchi, Gumucio, Yamada *et al.* 1986).

The rat NPY gene has also been cloned and shows a very high degree of homology with that of the human (Larhammar, Ericsson & Persson, 1987). Larhammar and co-workers have also—very recently—cloned both the chicken cDNA and the corresponding gene (see Minth & Dixon, 1989), which again are highly conserved at both the nucleotide level and amino acid sequence. This underlines the very high degree of conservation of NPY, and of the gene encoding the peptide, throughout evolution.

Also of interest in this regard are the predicted sequences of the flanking peptide (CPON), which are highly conserved between the human and rat structures. There are only two, conservative, amino acid substitutions: the rat sequence has alanine at position 19 in place of the valine of the human structure, and serine is substituted for the human alanine at position 28 in the rat (Allen, 1989). The presence of this peptide has been demonstrated by specific RIA in many different tissues, such as the heart, adrenals, hypothalamus and other parts of the brain (Allen, Polak & Bloom, 1985; Gulbenkian, Wharton, Hacker *et al.* 1985). It is unclear whether it has any physiological function(s), or is merely a byproduct of NPY biosynthesis. However, CPON-ir has been identified in plasma (Allen, Yeats, Causon *et al.* 1987), indicating that it is released. Moreover, the remarkable conservation of the CPON sequence across pecies—in contrast to the diversity reported between species of the eptide predicted to flank PP (Allen, 1989)—suggests that this peptide may ave an as yet unidentified regulatory role.

.3.4. Anatomical localization

Neuropeptide Y has been located using RIA and immunohistochemical echniques throughout the central, peripheral and enteric nervous ystems. It is one of the most abundant peptides in the central nervous ystem—perhaps even more so than somatostatin. NPY-ir neurones are videly distributed in all areas of the neocortex, hippocampus, basal orebrain, striatum, limbic structures such as the amygdala, the ypothalamus and the brain stem. In particular, NPY has been measured n very high concentrations in rat and human hypothalamus (Adrian, llen, Bloom *et al.* 1983; Allen, Adrian, Allen *et al.* 1983; Chronwall, DiMaggio, Massari *et al.* 1985). Additionally, NPY has been demonstrated

in most organ systems including the gut, heart, lungs, adrenals, pancreas, thyroid, and the vasculature—both peripheral and cerebral—and is also localized to the peripheral nervous system and the intrinsic innervation of many organs; it is found in reproductive tissues such as the ovary and placenta, and also in follicular fluid and plasma (e.g., Edvinsson, Emson, McCulloch *et al.* 1983; Lundberg, Terenius, Hökfelt & Goldstein, 1983; Sundler, Moghimzadeh, Håkanson *et al.* 1983; Gu, Polak, Allen *et al.* 1984; Lundberg, Hökfelt, Hemsén *et al.* 1986; McDonald, Dees, Ahmed *et al.* 1987; Petraglia, Calza, Giardino *et al.* 1989; Petraglia, Coukos, Battaglia *et al.* 1989; Jørgensen, O'Hare & Andersen, 1990; see also McDonald, 1988).

NPY is also of particular interest because of its co-existence with other classical neurotransmitters (see Everitt & Hökfelt, 1989). In the PNS, NPY has been co-localized with noradrenaline and adrenaline (Lundberg, Terenius, Hökfelt *et al.* 1982; Lundberg *et al.* 1983; Ekblad, Edvinsson, Wahlestedt *et al.* 1984). In the CNS, NPY-ir has been demonstrated in the noradrenergic A1 and adrenergic C1 and C2 cell groups in the brain stem and in many of the noradrenergic cell bodies of the A6 group in the locus coeruleus (Hökfelt, Lundberg, Tatemoto *et al.* 1983; Everitt, Hökfelt, Terenius *et al.* 1984; Everitt & Hökfelt, 1989). Sawchenko, Swanson, Grzanna *et al.* (1985) have also demonstrated extensive co-localization of NPY in adrenergic and noradrenergic neurones projecting to the PVN which arise from the C1, C2, C3 and A1 cell groups in the brain stem.

There are, however, other NPY-containing systems in the brain which lack noradrenaline. Bai, Yamano, Shiotani *et al.* (1985) have demonstrated an important projection of NPY neurones from the arcuate nucleus to the PVN and the dorso-medial hypothalamus. NPY-ir has also been shown in neurones throughout the neocortex which contain either somatostatin (see Everitt & Hökfelt, 1989) or GABA (Hendry, Jones, DeFelipe *et al.* 1984).

Neuropeptide Y synthesis and activity is not confined to cells of the nervous systems, however. Ericsson, Larhammar, McIntyre & Persson (1987) have shown that NPY and NPY mRNA are also found in tissues of the immune system. A combination of *in situ* hybridization and immunocytochemistry has shown NPY peptide and mRNA for NPY in the spleen, bone marrow and in the platelet-forming megakaryocytes (Larhammar, Ericsson & Persson, 1987; Ericsson, Schalling, McIntyre *et al.* 1987). As NPY is a potent vasoconstrictor (see below), it is possible that NPY is released during platelet aggregation resulting in a long-lasting vasoconstriction. Also of interest in this connection is that the levels of

megakaryote-derived NPY are much higher in some auto-immune mice, emphasizing the connection between the neuronal and immune systems.

2.3.5. General physiological aspects of NPY

There is physiological evidence that NPY acts at several loci. In vascular tissue, for instance, it has a direct vasoconstrictor effect and enhances the action of other vasoconstrictive agents such as noradrenaline, adrenaline and histamine (Lundberg *et al.* 1982; Lundberg & Tatemoto, 1982; Edvinsson, Ekblad, Håkanson & Wahlestedt, 1984; Ekblad *et al.* 1984). In contrast, NPY inhibits the electrically stimulated contractions of vas deferens and urinary bladder, without affecting the responses of these tissues to exogenously applied transmitter substances, suggesting that NPY also has a presynaptic action (Allen, Adrian, Tatemoto *et al.* 1982; Ohhashi & Jacobowitz, 1983; Lundberg, Hua & Franco-Cereceda, 1984). In vas deferens, NPY has been shown to inhibit the electrically stimulated release of noradrenaline, also by a presynaptic mechanism (Lundberg & Stjarne, 1984). NPY also acts presynaptically in the CA1 region of the hippocampus to reduce excitatory input to the pyramidal neurones (Colmers, Lukowiak & Pittman, 1985; 1987). This particular action was a longer term effect than the effects produced by other peptides such as somatostatin and oxytocin (Pittman & Siggins, 1981; Muhlethaler, Charpak & Dreifuss, 1984).

NPY has been observed to have long-lasting effects both on the physiology of peripheral tissues (Lundberg *et al.* 1982; Edvinsson *et al.* 984; Lundberg & Stjarne, 1984) and of the CNS (Carter, Vallejo & ,ightman, 1985; Potter, 1985; Stanley & Leibowitz, 1985). As NPY is co-tored with other neurotransmitters such as noradrenaline and GABA, his suggests that the longer term effects of NPY may interact with the horter term actions of the neurotransmitters as has been suggested for ther co-localized peptides and transmitters (Hökfelt, Johansson, ,jungdahl *et al.* 1980; Hökfelt, Johansson & Goldstein, 1984; Hökfelt, 'veritt, Meister *et al.* 1986).

A role for NPY has been suggested in sympathetic neurotransmission, s a selective increase in plasma NPY levels was observed during hysiological activation of this system by physical exercise (Lundberg, Martinsson, Hemsén *et al.* 1985). NPY increased in parallel with plasma oradrenaline, which suggests that the origin of NPY is neural in this case, ather than adrenal, and supports the hypothesis that NPY is co-secreted vith noradrenaline.

In the CNS, exogenously applied NPY has many physiological effects which appear to be independent of the action of noradrenaline. For instance, intracerebroventricular (i.c.v.) injection of NPY in the anaesthetized rat causes bradypnoea, hypotension and synchronization of electroencephalograph activity (Fuxe, Agnati, Härfstrand *et al.* 1983). Microinjection of NPY into the PVN or into the third ventricle causes a potent increase in feeding behaviour (Clark, Kalra, Crowley & Kalra, 1984; Stanley & Leibowitz, 1984; 1985). The hyperphagic effect appears to be due to an enhancement of carbohydrate ingestion (Stanley, Daniel, Chin & Leibowitz, 1985) which is of interest as i.c.v. injection of NPY stimulates insulin secretion for up to 2 h after injection (Moltz & McDonald, 1985). These authors also reported that NPY has direct inhibitory effects on glucose-stimulated insulin release from the perfused pancreas *in vivo* and from isolated pancreatic islet cells *in vitro*. Intracerebroventricular injection of NPY also enhances memory retention and recall in untrained mice (Morley & Flood, 1989). Although feeding enhances memory (Flood, Smith & Morley, 1987), this response is not mediated via a stimulation of food intake.

NPY has been implicated as a chemical messenger in the light-dark cycle entrainment of circadian rhythms. Albers & Ferris (1984) have shown that microinjection of NPY into the SCN of the hamster causes a phase shift in the circadian activity rhythm. Injection of NPY into the SCN in the 12 h before the onset of activity in free-running (constant light) hamsters advanced the phase, but phase-delayed the activity cycle when injected in the 12 h after the onset of activity. A shift in free-running activity rhythms had previously been observed when aPP was injected into the SCN (Albers, Ferris, Leeman & Goldman, 1984); however, the physiological ligand is most likely to be NPY. The anatomical NPY-ir neuronal pathway subserving the effect projects from the intergeniculate leaflet and ventral lateral geniculate nucleus of the thalamus, thus providing another pathway for the transmission of visual information (Card & Moore, 1982; Moore, Gustafson & Card, 1984; Harrington, Nance & Rusak, 1985).

2.3.6. NPY and neuroendocrine function

Nerve fibres immunoreactive for NPY are especially abundant in the preoptic area, paraventricular nucleus, mediobasal hypothalamus and other hypophysiotrophic areas of the brain (Everitt, Hökfelt, Terenius *et al.*

1984). In particular, there is immunohistochemical evidence indicating that NPY fibres are in close apposition to LHRH cell bodies in the area of the OVLT (Guy, Li & Pelletier, 1988). NPY-ir has been localized in axons and axonal terminals forming synaptic connections with ACTH-ir neuronal perikarya and dendrites in the arcuate nucleus (Csiffáry, Görcs & Palkovits, 1990). NPY neurones also project from the arcuate nucleus to the PVN (Bai, Yamano, Shiotani *et al.* 1985) where NPY nerve terminals have been visualized in the immediate vicinity of CRH nerve cell bodies (Wahlestedt, Skagerberg, Ekman *et al.* 1987) and NPY-ir axons have been shown to establish synaptic specializations with parvocellular neurones expressing CRH-ir (Liposits, Sievers & Paull, 1988). Thus there is morphological evidence that NPY may be important in the regulation of the secretion of both LHRH and CRH. There is evidence *in vivo* and *in vitro* that this is indeed the case.

In the rat and rabbit, NPY has been shown to exhibit modulatory activity at both the adenohypophysial and the central levels of the hypothalamo-pituitary-ovarian axis and has a biphasic action on LH secretion, ovarian factors being critical in the direction of the changes. Centrally administered NPY stimulates the secretion of LH in intact female rats (Kalra & Crowley, 1984; Crowley, Tessel, O'Donohue *et al.* 1985). However, LH secretion is inhibited by central NPY in ovariectomized female and intact male rats (Kalra & Crowley, 1984; Crowley *et al.* 1985; Kerkerian, Guy, Lefèvre & Pelletier, 1985; McDonald, ᵀumpkin, Samson & McCann, 1985), an effect which appears to be due to ɹ inhibition of pulsatile LHRH secretion from the hypothalamus ʌcDonald, Lumpkin & DePaolo, 1989). Push-pull perfusion studies in ıe rabbit and experiments in the rabbit and the rat *in vitro* also support ıe hypothesis that the effects on LH release are due to stimulatory or ıhibitory effects on hypothalamic LHRH release (Crowley & Kalra, 1987; horram, Pau & Spies, 1987; 1988). The biphasic effects of NPY on LH ılease are identical to those observed with noradrenaline (e.g., see Kalra Crowley, 1984; McDonald *et al.* 1985), which is of interest in the light of ıe co-localization of NPY and noradrenaline described above.

Central administration of NPY increases ir-CRH in the hypothalamus the rat (Haas & George, 1987). Furthermore, NPY evokes the release of RH from rat hypothalamus *in vitro* in a dose-dependent manner saragarakis, Rees, Besser & Grossman, 1989). Moreover, injection of PY into the SON increases circulating levels of vasopressin (Willoughby

& Blessing, 1985). In addition, direct injection of NPY into the PVN of the anaesthetized rat increases plasma ACTH levels (Wahlestedt, Skagerberg, Ekman *et al.* 1987) as does intracerebroventricular administration of NPY in the rat and dog (Härfstrand, Eneroth, Agnati & Fuxe, 1987; Inoue, Inui, Okita *et al.* 1989). NPY also potentiates the effectiveness of CRH as a secretagogue for ACTH in the dog, acting at sub-threshold doses in synergism with CRH (Inoue *et al.* 1989). Thus NPY appears to have a stimulatory role on the hypothalamo-pituitary-adrenal axis, acting at the level of the hypothalamus.

Although the neuroendocrine aspects of NPY still require much investigation compared with the vast literature on the cardiovascular effects, it is clear that NPY has a major potential role in the regulatory control and/or modulation of the reproductive and stress axes.

3. General materials and methods

The materials and methods described in the following chapter are common to all experiments performed. Those details unique to a particular experiment are described in the relevant chapters.

3.1. Animals and management

Adult Scottish Blackface ewes, obtained from the Macaulay Land Use Research Institute flock at Sourhope Research Farm, Yetholm, Roxburghshire, were used for the majority of the studies described in this thesis. Finn x Dorset crossed ewes from the Institute of Animal Physiology and Genetics Research farm at Blythbank, Peeblesshire, were used for some of those reported in Chapters 8 and 9.

For the majority of experiments, animals were housed indoors in individual pens (1.5 m x 2 m) in the University of Edinburgh Marshall Building at Dryden-Mountmarle, near Roslin, Midlothian. The animals were fed 500 g supplementary pelleted feedstuff (Moredun Nuts, Dalgety Agriculture, Bristol) each morning and had access to hay and water *ad libitum*. For the infusion experiments described in Chapter 6 and the peripheral immunization experiments in Chapter 9, sheep were kept in approved restraint crates which allowed them room to stand up and lie down and access to food and water, but not to turn around. Experiments were performed under light-controlled conditions as described in each chapter.

All experiments were approved by the Home Office under the terms of the Animals (Scientific Procedures) Act 1986; Project Licence PPL 60/00612, Personal Licence PIL 60/01087.

.2. Surgical preparation of animals

.2.1. Anaesthesia

Anaesthesia was induced with Saffan (9 mg/ml alphaxolone, 3 mg/ml alphadolone acetate, solubilized in saline by 20% (w/v) polyoxyethylated

castor oil, Glaxovet, Harefield, Middlesex). Depending on the size of the animal, 18–25 ml was injected intravenously. The ewe was then intubated with an endotracheal tube 8.5–9.5 mm in diameter, and connected to a portable anaesthetic machine (Cyprane, Keighley, Yorkshire). Anaesthesia was maintained with ~3–4% halothane (Fluothane, ICI Pharmaceuticals, Macclesfield, Cheshire) in a nitrous oxide/oxygen mixture (both ~0.8 l/min).

3.2.2. Ovariectomy

Anaesthesia was induced and maintained as described above. The animal was then secured on the operating table in the supine position. The wool over the abdomen was clipped and the area exposed cleansed and disinfected with surgical scrub and antiseptic solutions (Betadine, Napp Laboratories, Cambridge).

A midline incision (~12 cm) was then made in the skin overlying the abdomen, starting just above the mammary gland and avoiding the mammary vein. The peritoneum was exposed by blunt dissection with forceps and an incision made just adjacent to the midline ridge. Great care was taken to prevent accidental damage to the intestine underlying the site of incision and to avoid the abdominal musculature. The uterus was then located and the first ovary clamped off with curved forceps. The tissue between the ovary and the uterus was tied off in two halves with Mersilk (size 4 metric, Ethicon, Edinburgh) using a locking stitch and then reef knot, the grip of the clamp being released as the ligatures were tied. ıe ovary was then sliced off with a scalpel, ensuring complete removal all ovarian tissue. This procedure was then repeated for the contra-teral ovary.

After careful replacement of the uterus, the peritoneum was sewn up ith individual stitches of sterile chromic catgut (size 6 metric, Ethicon) ›proximately every 1 cm using a round-bodied needle, taking care to ˈoid the intestine underneath. The wound was treated with an ıtibacterial spray containing Bacitracin, Neomycin and Polymyxin ˈolybactrin, Wellcome, London) and closed with a running stitch rough the skin. Animals were usually alert and eating again within 20 in of removal from the operating table. Each animal received 4 ml reptopen (250 mg/ml procaine penicillin and 250 mg/ml dihydro-ɾeptomycin sulphate, Glaxovet) daily for 4 days post-operatively.

3.2.3. Intracerebroventricular cannulation

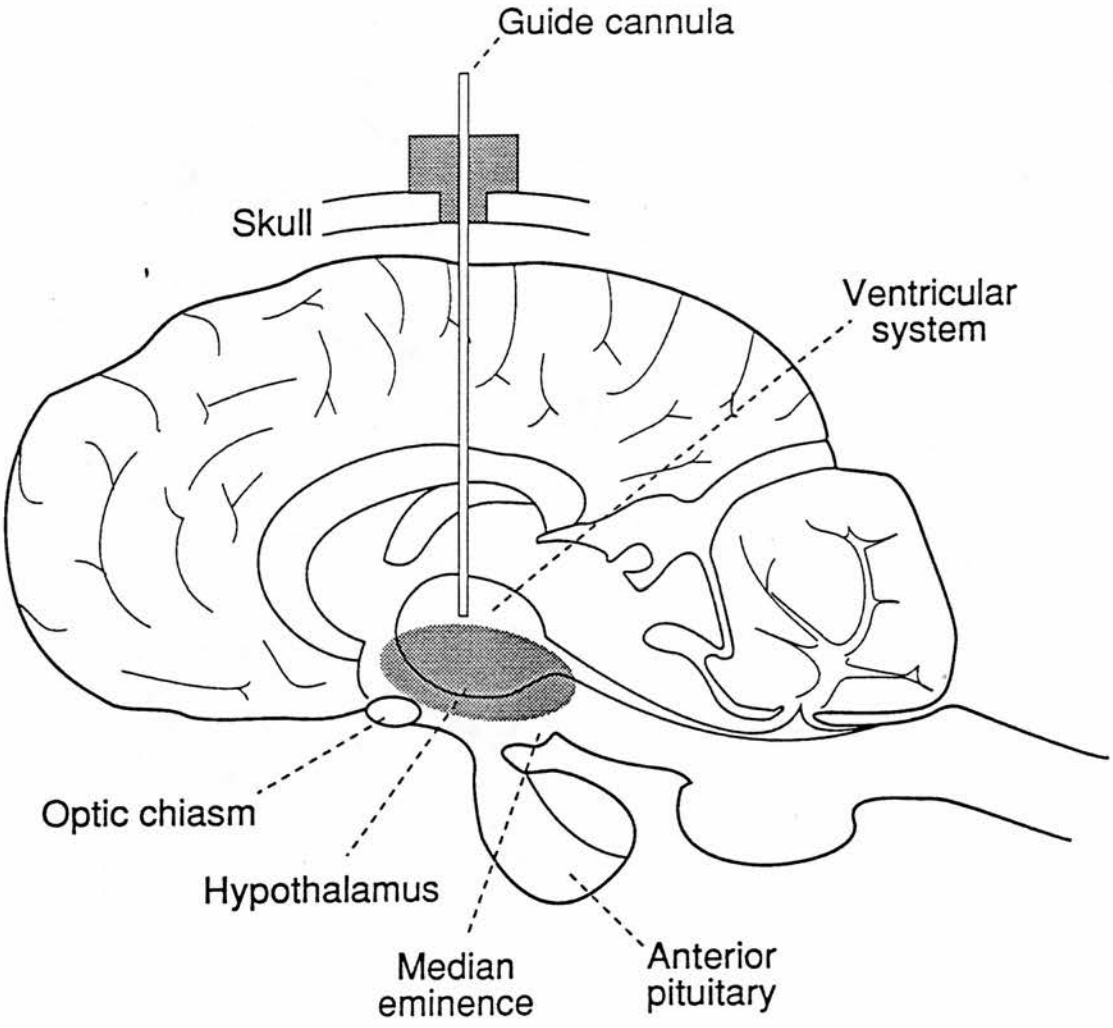
Anaesthesia was induced and maintained as described above. The animal was secured on the operating table in the prone position and its head secured firmly in a stereotaxic frame made in the Preclinical Veterinary Sciences workshop of the Royal (Dick) Veterinary School, University of Edinburgh. The skin overlying the superior aspect of the head was shaved of wool and cleansed and disinfected with Betadine surgical scrub and antiseptic solutions.

Using aseptic technique throughout, a midline incision was made (~8 cm), starting between the horns and passing rostral towards the gristle pad. The skin was retracted and the skull surface exposed. Bregma was identified and marked with a small depression using a dental drill (Osteon, Amsco Hall Surgical). A bore hole, 11 mm in diameter, was made through both tables of the skull at bregma with a purpose-built surgical drill (Aesculap-Werke AG, Tuttlingen, FRG) using this mark as a guide. The drill was designed to stop immediately upon reaching the dura mater. The sliver of bone remaining was removed with a fine pair of tweezers thus exposing the sagittal sinus.

With the stereotaxic atlas drawn by Dr Ph. Richard (1967) for the Préalpes-du-Sud breed as an approximate guide, a sterile stainless steel cannula 45 mm long, fashioned from the tip of an 18-gauge spinal needle (Becton-Dickinson, Rutherford, NJ, USA) was then inserted at bregma ırough the brain tissue to a depth of approximately 20 mm. In order to void piercing the sinus, the point of insertion was just lateral to this lood vessel and the cannula was angled so as to reach the midline and ıerefore the third ventricle (see Figure 3.1). This usually produced a ɔpious flow of cerebrospinal fluid up the cannula which was stemmed by ıe insertion of a 20-gauge stylette into the spinal needle. Immediately fter the insertion of the cannula into the brain tissue, the base of the drill ole was filled with absorbable gelatin sponge (Sterispon, Allen & lanburys Ltd, Greenford, Middlesex), to form a stable matrix for sealing ıe cavity. The hole was then filled with dental cement (Formatray, Kerr urope, Basel, Switzerland).

The top of a 500-ml screw-top polyethylene bottle (BDH, Poole, Dorset) ras cut to shape to form a protective covering over the skull wound and ɛcured to the skull with self-tapping stainless steel screws just rostral and ıst caudal of the drill hole. The screw heads were covered and the space

Figure 3.1. Schematic diagram showing the placement of the third ventricular guide cannula relative to the ventricular system and the hypothalamus in the ewe. This cannula allows easy access to the cerebrospinal fluid and therefore to periventricular structures involved in the control of the anterior pituitary gland. The top of the cannula is protected by a polyethylene cap (not shown). Not to scale.


Guide cannula
Skull
Ventricular system
Optic chiasm
Hypothalamus
Median eminence
Anterior pituitary

between cannula and cap filled with dental cement. Finally, the wound was treated with antibacterial spray (Polybactrin) and closed with sutures. A rubber disc, cut to fit, was placed inside the cap to keep the cannula assembly clean.

The animals were invariably alert, standing and eating within 1 hour. As above, all received 4 ml Streptopen daily for 4 days post-operatively. The Finn x Dorset ewes (Chapters 8 and 9) were also given an anti-inflammatory analgesic. Each animal received 3 ml Tomanol (240 mg/ml isopyrin and 130 mg/ml phenylbutazone sodium, Intervet Laboratories, Cambridge) by slow intravenous injection daily for 2 days post-operatively.

3.3. Experimental procedures

3.3.1. Blood sampling

During intensive sampling regimes, ewes were chronically cannulated. On the day before each sampling period, a cannula (Braunüle, Braun AG, Melsungen, FRG) was inserted into the jugular vein, stitched securely to the neck close to the point of entry and attached to a three-way stopcock (Connecta, Viggo AB, Helsingborg, Sweden) via 60 cm of polythene manometer connecting tubing with luer lock fittings (Portex, Hythe, Kent). The cannula was kept patent with heparinized saline [20 units heparin (Leo Laboratories, Aylesbury, Bucks)/ml 0.9% (w/v) NaCl (Travenol Laboratories, Thetford, Norfolk)]. Blood samples (1–3 ml) were collected at)-min intervals for 11–12 h from 08.00 h. Samples were withdrawn using ml sterile syringes and immediately placed in 7-ml polystyrene tubes arstedt, Leicester) containing heparinized beads (Lithium Heparin ırrier Beads, Sarstedt). The sampling line was immediately flushed with)proximately 2 ml heparinized saline. Samples were spun at 3 000 rpm 510 *g*) (MSE Chilspin 2, Fisons Instruments, Crawley, Sussex) for 15 min ıd the separated plasma decanted into 2-ml plastic sample tubes (Teklab, ıcriston, Durham) which were then stoppered and stored at –20 °C until sayed.

3.2. Intracerebroventricular injections

most cases, two i.c.v. injections were given, the first after an initial 4-h ıntrol period, the second 1.5 h later. Immediately before each injection, e stylette was removed from the guide cannula and a 20-gauge injector

needle, connected to a length of PE 100 tubing (Becton-Dickinson) containing the solution to be injected, was lowered to the required depth, i.e., just beyond the end of the guide cannula. The peptides, dissolved in sterile 0.9% saline immediately prior to injection as required, were delivered by gravity flow into the third ventricle in a volume of 50 µl. The injection tubing was usually back-loaded with enough doses of the peptide for 2–3 animals. After use, the needles and tubing were flushed thoroughly with sterile saline and 70% ethanol. In treatments where animals received both LHRH and LHRH antagonist, the antagonist was delivered (i.c.v.) 15 min before i.c.v. injection of LHRH. The order of the various injections was based on a cross-over design where possible, with each ewe serving as its own control and receiving each treatment.

3.4. Neuropeptides and drugs

Synthetic LHRH and the LHRH agonist, Buserelin, ([*D*-Ser-(tBu)^6, Pro^9]-LHRH-(1–9)-nonapeptide-ethylamide) were generous gifts from Dr J. Sandow (Hoechst AG, Frankfurt, FRG). The LHRH antagonist, Detirelix, ([N-Ac-*D*-Nal(2)^1, *D*-*p*Cl-Phe^2, *D*-Trp^3, *D*-hArg-(Et$_2$)^6, *D*-Ala^{10}]-LHRH) was a generous gift from Dr B.H. Vickery (Syntex, Palo Alto, CA, USA). I should like to thank Dr Hamish Fraser for his kind help in obtaining these peptides. Synthetic ovine corticotrophin-releasing hormone (oCRH) was purchased from Bachem UK Ltd (Saffron Walden, ssex), naloxone (as N-allylnoroxymorphone-hydrochloride) and cortisol s hydrocortisone sodium semisuccinate) from Sigma (Poole, Dorset), and nthetic human neuropeptide Y (hNPY) from Cambridge Research iochemicals (Harston, Cambridge).

5. Radioimmunoassay procedures

5.1. Luteinizing hormone

oncentrations of LH in plasma were measured by the specific double-ıtibody radioimmunoassay of McNeilly, Jonassen & Fraser (1986).

uffer Assay buffer (0.1% BSA 0.075 M phosphate-buffered saline, pH 5) (0.1% BSA/PBS) was made by dissolving 8.76 g NaCl and 2.0 g bovine bumin (Sigma RIA grade, fraction V) in 300 ml 0.5 M phosphate buffer ıH 7.5) and 1700 ml distilled water. The pH was then re-adjusted to 7.5.

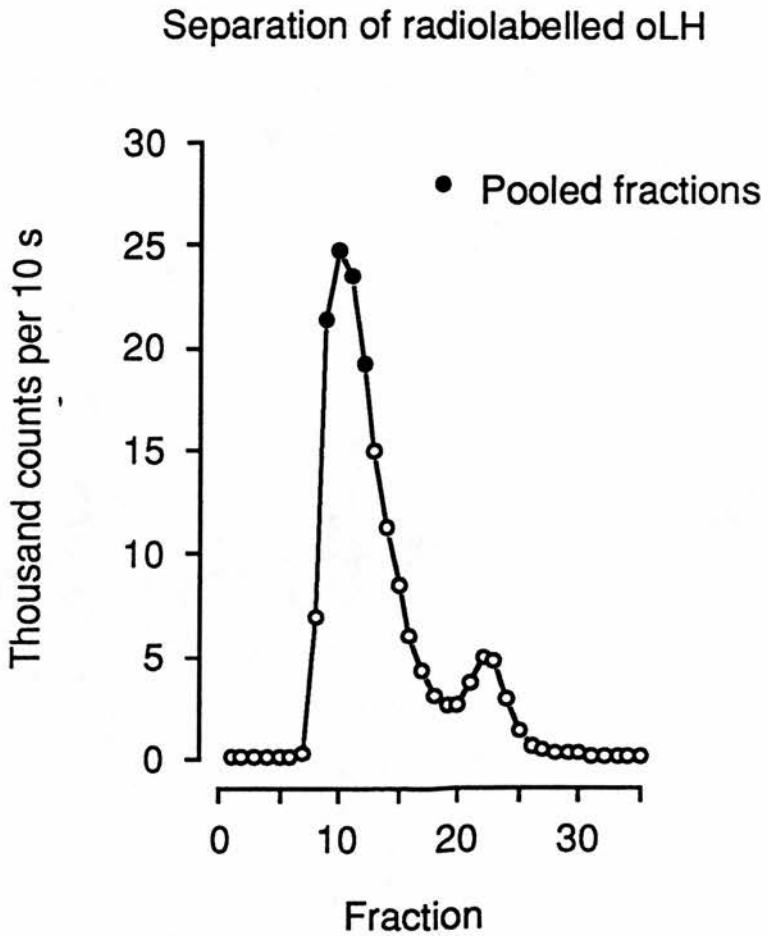
Antibody The assay used a rabbit antiserum (R 29) raised by Dr Alan McNeilly against ovine LH (NIH-oLH-S19) at a final dilution of 1: 600 000. This shows < 0.1% cross-reaction with ovine FSH, LH α-subunit or prolactin or bovine TSH (McNeilly *et al.* 1986).

Radioiodination Highly purified ovine LH (LER-1056-C2)—a generous gift from Dr L.E. Reichert Jr., Albany Medical College of Union University, Albany, NY, USA—was used to prepare radioiodinated LH label. 500 μl of thiomersal-free 0.075 M phosphate-buffered saline (PBS) was added to 100 μg LER-1056-C2 to make a stock solution, which was stored at –20 °C in aliquots of 25 μl, each containing 5 μg hormone.

The lactoperoxidase method was used to prepare the label. 5 μl (500 μCi) of $Na^{125}I$ (Amersham International, Aylesbury, Bucks) and 5 μg of lactoperoxidase (Sigma) in 10 μl thiomersal-free PBS were added to 5 μg LER-1056-C2. 10 μl hydrogen peroxide (10 μl H_2O_2/150 ml distilled water) was then added to start the reaction. After 20 sec, the reaction was stopped by the addition of 10 μl of a 1.0 mg/ml solution of 2-mercaptoethyl-ammonium chloride (cysteamine hydrochloride) in distilled water. 1 ml of thiomersal-free 1% BSA/PBS was added to increase the volume of the reaction mixture. The resulting 'cocktail' was subjected to gel filtration on a Sephadex G-100 column (G-100 from Pharmacia AB, Uppsala, Sweden; column from Amicon, Stonehouse, Gloucestershire). The gel was allowed to swell in thiomersal-free PBS that had first been allowed to equilibrate to room temperature. After the column was poured and allowed to pack own, it was coated with thiomersal-free 1% BSA/PBS. The iodination ocktail was added to the column and eluted with 0.1% BSA/PBS. The lutate from the column was collected in 8-ml polystyrene tubes using a rogrammable electronic fraction collector (Gilson model 203, Middleton, VI, USA) and the radioactivity in each fraction monitored (Mini-assay ype 6-20, Mini Instruments, Burnham-on-Crouch, Essex). The first peak luted from the column was purified radiolabelled LH tracer, free from amaged hormone and unbound ^{125}I (see Figure 3.2). The four fractions of his peak containing the highest radioactivity were pooled and stored in liquots of 250 μl at –20 °C until required. Incorporation of ^{125}I into the rotein peak was typically 70–80% and the specific activity 30–50 μCi/μg LH.

When 1 mCi (10 μl) of ^{125}I was used to prepare radioiodinated LH, rapid oss of binding occurred and quality control results were altered. The kely reason for this was that the specific activity of the label was too high,

Figure 3.2. Radioactivity in fractions eluted from Sephadex G-100 column after radioiodination of oLH, showing incorporation of ^{125}I into the first (protein) peak. The fractions that were pooled and used as ^{125}I- oLH tracer are also shown.


Separation of radiolabelled oLH
Pooled fractions
Thousand counts per 10 s
30
25
20
15
10
5
0
0
10
20
30
Fraction

causing degradation of the hormone. Therefore, 500 μCi was used for all iodinations.

Standards Each assay contained at least a standard curve at the beginning and end, with further curves if required. Each comprised a set of standard points double-diluted from 50–0.1 ng/ml. Standards were kindly supplied by the National Hormone and Pituitary Program of the NIDDK, Bethesda, MD, USA. NIH-LH-S18 was used for all experiments apart from those in chapters 8 and 9 where NIH-LH-S23 was used as standard. In our hands, we have found 1 ng/ml NIH-LH-S23 ≈ 2 ng/ml NIH-LH-S18 (see Figure 3.3). Results are expressed in terms of ng NIH-LH-S18/ml unless otherwise indicated.

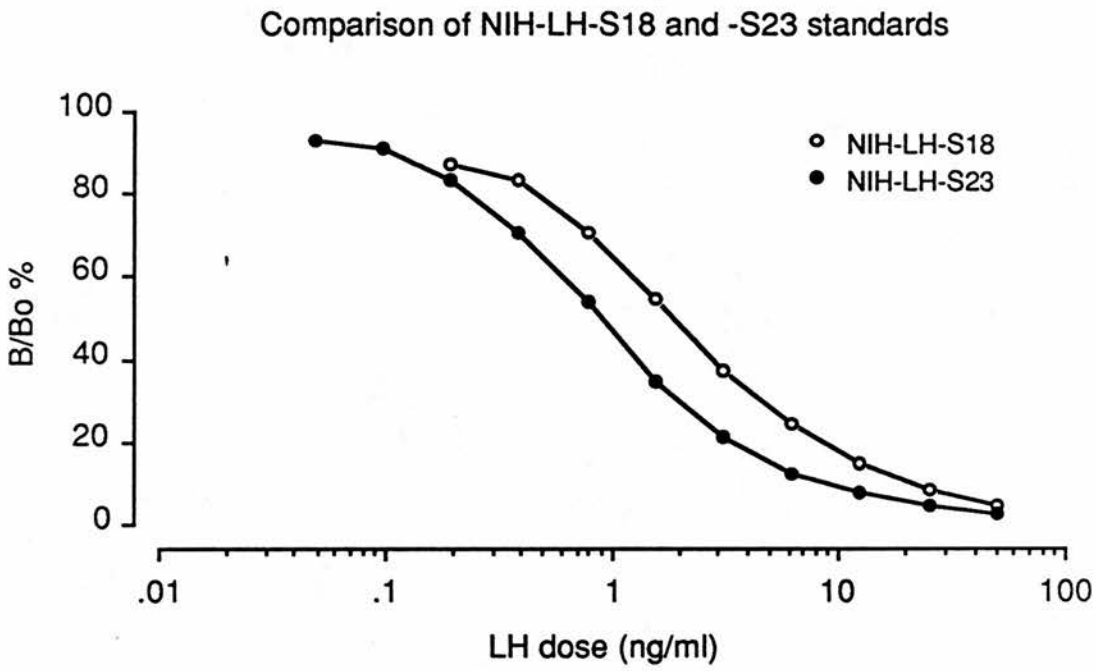
Separation The bound and free fractions were separated by liquid phase second antibody precipitation using donkey anti-rabbit serum and normal rabbit serum obtained from the Scottish Antibody Production Unit (SAPU) (Carluke, Lanarkshire).

Procedure Samples, standards and quality control plasma samples were dispensed and diluted as appropriate using a programmable electronic pipetting station (Hamilton Microlab M, Hamilton Bonaduz AG, Switzerland). 100 μl of standard or sample (after dilution if required) was added to 200 μl assay buffer in 3-ml polystyrene tubes. Each standard curve was set up in triplicate and samples assayed in duplicate. The anti-serum (R 29) was diluted in assay buffer to give a dilution of 1: 120 000. 100 μl of this solution was then added to each tube. In addition to standards as above, a set of tubes including no 'cold' hormone, to assess undisplaced binding of the antibody to the labelled trace and a set of tubes containing no antibody and no cold hormone, to assess the non-specific binding, were included. These tubes received buffer in place of the standard and/or antibody. All tubes were vortexed and incubated at 4 °C for 24 h.

Radiolabelled hormone was then added to all tubes. The stored label was diluted in assay buffer to give approximately 15 000 cpm/100 μl and 100-μl aliquots added to each tube. A set of tubes containing trace alone was also prepared to determine the total number of counts added to each tube. All tubes were again vortexed and incubated at 4 °C for another 24 h.

All tubes then received 100 μl of normal rabbit serum, diluted 1: 800 in assay buffer, followed by 100 μl of a 1: 32 dilution of second antibody (donkey anti-rabbit serum) to precipitate the bound fraction. Tubes were again vortexed and incubated overnight at 4 °C.

Figure 3.3. Comparison of typical NIH-LH-S18 and NIH-LH-S23 standard curves, showing parallelism of standards. Data shown is after subtraction for non-specific binding.


Comparison of NIH-LH-S18 and -S23 standards
100
80
60
40
20
0
B/Bo %
NIH-LH-S18
NIH-LH-S23
.01
.1
1
10
100
LH dose (ng/ml)

1 ml of 0.9% saline was added to all tubes (except total counts) which were immediately centrifuged at 3 000 rpm (2 110 *g*) for 30 min at 4 °C (Sorvall Omnispin R, DuPont, Wilmington, DE, USA). The supernatant was then decanted and the precipitate counted for 60 s on a γ-counter (1261 Multigamma, LKB Wallac OY, Turku, Finland).

Sensitivity The lower limit of sensitivity of the assay, defined as the lowest standard to depress the binding by more than two standard deviations from the zero binding level, was 0.2 ng/ml using NIH-LH-S18 as a reference standard. The intra- and interassay coefficients of variation assessed using three pooled plasma samples containing 0.95, 3.92 and 6.58 ng/ml, were 7.3 and 15.9%, 6.1 and 10.1% and 6.2 and 13.2% respectively.

3.5.2. Follicle-stimulating hormone

The concentration of FSH was measured in plasma using the double-antibody radioimmunoassay described by McNeilly, McNeilly, Walton & Cunningham (1976). except that NIAMDD-anti-oFSH-1 was used as first antibody.

Briefly, duplicate 150-µl aliquots of sample, diluted as required, or triplicates of standard (0.5–125 ng/ml NIH-FSH-S14, NIDDK) were added to 150 µl assay buffer (0.1% BSA/PBS). Anti-ovine FSH antiserum (NIAMDD anti-oFSH-1), raised in a rabbit and obtained from NIH, was diluted 1: 12 000 in assay buffer and 50 µl added to all tubes apart from NSBs. Tubes were vortexed and incubated at 4 °C for 24 h.

Radiolabelled FSH was prepared by the lactoperoxidase method scribed above from purified ovine FSH (NIDDK-oFSH-I-1) obtained)m NIH. Tracer was diluted in assay buffer (15 000 cpm/50 µl) and 50 µl ded to all tubes which were then vortexed and incubated for a further h at 4 °C. Tubes then received second antibody as above and were :ubated overnight, followed by centrifugation and counting.

Assay sensitivity was 2 ng NIH-FSH-S14/ml; the intra- and interassay efficients of variation were both less than 10%.

i.3. Prolactin

olactin was measured in plasma by the double-antibody radioimmuno-say of McNeilly & Andrews (1974).

Briefly, samples were assayed in duplicate in a volume of 30 µl, diluted required. 30 µl of sample or standard (0.4–200 ng/ml NIH-oPRL-S15) is added to 100 µl assay buffer (1% BSA/PBS). The anti-ovine prolactin

antiserum used (#50) was raised by Dr Alan McNeilly in a rabbit and added at a dilution of 1: 128 000 in a volume of 100 µl. 100 µl radiolabelled prolactin, prepared by the lactoperoxidase method from purified ovine prolactin (NIADDK-oPRL-19) supplied by NIH, was added to each tube and the assay incubated at 4 °C for 24 h. Tubes then received second antibody as previously described, were incubated overnight and then centrifuged and counted.

Assay sensitivity was 0.8 ng NIH-PRL-S15/ml; the intra- and interassay coefficients of variation were both less than 8%.

3.5.4. Cortisol

Plasma cortisol concentrations were measured by the double-antibody radioimmunoassay described by Porter, Lincoln & Naylor (1990) and in more detail below.

Buffer Assay buffer (0.1% gelatin 0.1 M phosphate-buffered saline, pH 7.4) was made by dissolving 22.92 g Na_2HPO_4, 5.22 g $NaH_2PO_4.2H_2O$ and 18.0 g NaCl in 2 l distilled water. About 50 ml of the measured distilled water was set aside and warmed to 37 °C to dissolve 2.0 g gelatin. Once the gelatin was in solution, this was returned to the rest of the buffer and the pH adjusted to 7.4.

Extraction All samples from a particular treatment of an individual animal were extracted together and included in the same assay. Steroids were extracted from 100 µl plasma with 2.5 ml diethyl ether (BDH Analar grade). After vortexing for a minimum of 10 min, the aqueous phase was frozen in a dry ice/methanol bath and the organic phase decanted into new borosilicate tubes and evaporated to dryness under nitrogen. At this point, the dried-down tubes were stored, if required, at –20 °C for assay at a later date. The steroid fraction was then reconstituted in 500 µl assay buffer, vortexed thoroughly and left for at least 0.5 h at room temperature.

Correction was made for methodological losses in the extraction of each batch of samples. Six 100-µl aliquots of dexamethasone-suppressed sheep plasma, with 10 µl of label containing 15 000 cpm ^{125}I-cortisol added, were subjected to the same extraction procedures and the amount of label recovered was counted. The mean extraction efficiency was 75%.

Antibody Anti-cortisol antibody (S004-201), raised in a sheep against cortisol-3-(carboxymethyl)-oxime-BSA conjugate, was obtained from the Scottish Antibody Production Unit (Carluke, Lanarkshire). A stock solu-

tion of this antibody was made up in assay buffer to a dilution of 1: 10 and stored at –20 °C in aliquots of 100 µl. This was further diluted to a working dilution of 1: 8 000 immediately before use. Cross-reactivities of the antibody as quoted by the supplier are as follows: corticosterone, 0.18%; cortisone, 0.07%; 21-deoxycortisone, 0.30%; 11-deoxycorticosterone, 0.03%; 11-deoxycortisol, 0.58%; 17α-OH progesterone, 2.10%.

Radioiodination The cortisol for iodination was first conjugated to histamine by Ian Swanston using the mixed-anhydride method and stored dried down in glass test tubes at –20 °C in aliquots of 500 ng.

The cortisol-histamine conjugate, in its glass tube, was iodinated using the chloramine-T technique as follows. After drying down any residual ethanol from the conjugation reaction, the conjugate was solvated in 40 µl 0.05 M phosphate buffer for 20 min at room temperature. 10 µl (1 mCi) $Na^{125}I$ and then 10 µl chloramine-T (5 mg/ml 0.25 M phosphate buffer) were added and the mixture vortexed for 60 s. The reaction was stopped by the addition of 10 µl sodium metabisulphite (8 mg/ml 0.05 M phosphate buffer) and the mixture then counted to determine the total radioactivity present. 180 µl 0.05 M phosphate buffer was added and the steroid fraction extracted by vortexing for 60 s with 400 µl ethyl acetate. The two phases were allowed to separate and the (upper) steroid fraction transferred to a small glass test tube with a pasteur pipette. This was counted to determine the radioactivity in the steroid fraction. The labelled cortisol was then separated from the free ^{125}I in the steroid fraction by thin-layer romatography (Merck silica gel 60 F_{254} aluminium TLC plate, Merck AG, ırmstadt, FRG) using 150 ml toluene, ethanol and glacial acetic acid 5: 24: 1 v/v) as the solvent. After the plate had run for 1.5–2 h the dioactive bands were located using a geiger counter shielded with a lead t. The lower band (R_f ~0.35) was cut out into 1-cm pieces and placed in 5 ethanol in a glass-stoppered test tube at 4 °C. When required for assay, e aliquot of labelled cortisol was dried down in a beaker under nitrogen d reconstituted in assay buffer. Incorporation of ^{125}I into the steroid ıction was typically 30–35% and the specific activity of the labelled rtisol 0.6–0.8 µCi/100 µl.

For the cortisol assays in chapters 8 and 9, ^{125}I-radiolabelled cortisol ortisol-3-(carboxymethyl)-oximino-(2-[^{125}I]-iodohistamine)] was purased from Amersham. This increased the binding from 35% to 60%, but ıerwise did not alter any characteristic of the assay.

Procedure Cortisol for use as standard was obtained from Sigma (Poole, Dorset). A stock solution of 100 µg/10 ml assay buffer was made from a solution of 1 mg/ml cortisol in ethanol. Standards were prepared by double dilution in assay buffer from 8 000–15.6 pg/100 µl. Duplicate 100-µl aliquots of the extracted sample, reconstituted in 500 µl assay buffer as above, were incubated for a minimum of 4 h at room temperature, or overnight at 4 °C, with 100 µl anti-cortisol antibody and 100 µl ^{125}I-labelled cortisol (15 000 cpm/100 µl). The bound fraction was separated by liquid phase second antibody precipitation by incubating overnight at 4 °C with 100 µl donkey anti-sheep serum (1: 30) and 100 µl normal sheep serum (1: 600) (SAPU). 1 ml 0.9% saline/0.2% Triton X-100 was added to all tubes except total counts before centrifugation and counting as above.

Sensitivity The sensitivity of the assay was 31 pg/tube and the detection limit 1.55 ng/ml. Dexamethasone-suppressed sheep plasma with cortisol added at three concentrations was used for quality controls. The mean intra-assay coefficient of variation (CV) was 11.8% at a mean plasma cortisol concentration of 21.8 ng/ml, 7.7% at 65.2 ng/ml and 5.3% at 228 ng/ml for triplicates of three quality control plasma pools; the interassay CVs were 14.8%, 11.9% and 10.3% respectively for these same quality controls.

3.6. Data analysis

3.6.1. Data reduction

During counting (see above) the output from the Multigamma counter was written to a digital data logger (Datagrabber, Mutek, Box, Wiltshire). This allowed subsequent input to an assay calculation program written by Phil Taylor for the Apple Macintosh computer (AssayZap, Elsevier Biosoft, Cambridge). Essentially, this program uses a four-parameter weighted-regression model of the displacement curve. This starts from the two-parameter Logit-log model as a first estimate but then adjusts the asymptotes, slope and point of inflection (the four parameters) of the model curve reiteratively, giving more weight to the standard points with smaller variance, until the least squares fit is obtained. The program also stores information about assay history so that comparisons with previous curves, quality control samples, binding levels etc. are possible.

3.6.2. Data interpretation

For analysis of the data the sampling period was condensed, in most cases, by splitting it into three divisions. These corresponded to times before, during and after treatment. In all cases, these were less than one third of the total sampling time, due to the time taken to inject each animal. The injections had to be staggered, sometimes taking up to 45 min for all injections to be complete. For analysis of pulsatile data, pulses of LH were defined as occurring when the concentration of LH measured in one plasma sample was greater than the mean of the concentrations of the previous two samples by at least three times the CV of the assay. Pulse amplitude was calculated by subtracting the concentration at the onset of the pulse from the peak concentration; mean LH concentration by averaging the concentrations over the sampling window and pulse frequency by counting the number of peaks in the sampling window. The area under the curve was calculated by treating the area as composed of a series of trapezoids of the width of the sampling interval and calculating the sum of these small areas.

3.6.3. Statistical analysis

The effects of treatments were assessed statistically by analysis of variance either within- or between-groups and for repeated measures as appropriate (Winer, 1971) using a statistics program written for the Apple Macintosh mputer (CLR Anova, Clear Lake Research, Houston, TX, USA). Pairwise mparisons were made using Neuman-Keuls test of significance.

4. Effect of central administration of LHRH and LHRH analogues on LH secretion in the ovariectomized ewe

4.1. Introduction

The experiments described in this and the following two chapters were designed to address the concept of an autoregulatory mechanism, whereby hypothalamic neuropeptides alter the pattern of their own release. Such autoregulation was first postulated by Hyyppa, Motta & Martini (1971) for the putative follicle-stimulating hormone-releasing factor. They coined the term 'ultrashort'-loop feedback to differentiate this type of control from 'short'-loop feedback and 'long'-loop feedback. By these terms they meant, respectively, the control of secretion of the releasing factor by an intrahypothalamic action of the pituitary hormone (such as, in this case, FSH) and control by those hormones, such as the sex steroids, released from the peripheral target organs.

Autoregulatory mechanisms have been demonstrated for a number of hypothalamic releasing and inhibiting hormones. Somatostatin (Abe, o, Iwasaki *et al.* 1978; Lumpkin, Negro-Vilar & McCann, 1981), growth mone-releasing factor (Lumpkin, Samson & McCann, 1985) and einizing hormone-releasing hormone (Bedran de Castro, Khorram & Cann, 1985) all act within the brain to alter the release of anterior itary hormones in the opposite direction to their actions at the level of pituitary gland.

In the rat there is evidence, both *in vivo* and *in vitro*, that central ninistration of exogenous LHRH and LHRH agonists reduces einizing hormone (LH) secretion (Bedran de Castro *et al.* 1985; irguignon, Gerard, Debougnoux *et al.* 1987; DePaolo, King & Carrillo, 7; Sarkar, 1987). When LHRH was administered intracerebroventric- rly to ovariectomized rats, plasma levels of LH decreased, suggesting t exogenous LHRH might prevent the release of endogenous LHRH. physiological significance of endogenous LHRH in controlling LHRH

release has also been examined *in vitro*. When median eminence fragments were incubated in the presence of an LHRH antagonist ([*D*-pGlu^1, *D*-Phe^2, *D*-Trp^{3,6}] LHRH), basal secretion of LHRH increased in a dose-related manner (Valença, Johnston, Ching & Negro-Vilar, 1987), suggesting that endogenous LHRH may exert a tonic inhibitory influence on LHRH release. Thus, it is conceivable that centrally-released LHRH may be involved in the mechanisms governing the pulsatile secretion of LHRH, possibly through direct contacts between LHRH neurones (Leranth, Segura, Palkovits *et al.* 1985; Pelletier, 1987). The work described in this chapter was designed to expand on the findings in the rat and to investigate further the hypothesis of an LHRH 'ultrashort'-loop feedback.

As reviewed in more detail in Chapter 2, the pulsatile secretion of LHRH from the brain is thought to be brought about by the synchronous firing of LHRH neurones located within the hypothalamus. The resultant release of LHRH into the portal system is then responsible for the pulsatile secretion of LH from the pituitary gland. It is therefore possible to monitor LH pulses as an indicator of the activity of the hypothalamic LHRH pulse generator. The sheep is a particularly good animal model in which to study changes in the pulsatile secretion of LH over long periods of time as it has a large blood volume and well defined characteristics of LH release.

The following studies were therefore undertaken in the ovariectomized ewe to determine the effects of centrally administered LHRH and an LHRH antagonist on the pulsatile secretion of LH *in vivo*. Specifically, the aims were to investigate the effect of third ventricular injection of LHRH on the pulsatile properties of LH secretion, the effect of an LHRH antagonist on this response and also the possible role of endogenous LHRH in the feedback control of pulsatile LHRH secretion.

2. Experimental Design

Scottish Blackface ewes were housed in individual pens in the University of Edinburgh Marshall Building, under artificial lighting conditions (14 h light; 10 h dark) from October 1987 to January 1988. The sheep were fed concentrated pellets once a day; hay and water were available *ad libitum*.

Three to four weeks before experimentation, the animals were ovariectomized (Section 3.2.2) using aseptic conditions. Several days later

the sheep were anaesthetized again and a stainless steel cannula directed towards the third ventricle was implanted (Section 3.2.3).

The experiments described in this chapter were designed to investigate the concept of LHRH-autoregulation by means of the intracerebroventricular injection of LHRH and its analogues. Thus, central injections of LHRH with or without antagonist and of the antagonist alone were made and their effects on the secretion of LH studied. Also, in a preliminary experiment, LHRH agonist was administered centrally. Details of the agonist and antagonist are given in the previous chapter (Section 3.4). Two doses of LHRH were used; 2.1 pmol (2.5 ng) and 21 pmol (25 ng). The dose of antagonist was 69 pmol (100 ng) and those of the agonist, 0.8 pmol (1.0 ng) and 8.0 pmol (10 ng). All were delivered, under gravity, in a volume of 50 µl sterile saline. When the antagonist was given together with LHRH, 69 pmol antagonist was injected 15 min prior to each injection of 21 pmol LHRH.

On the day before each experiment began, a jugular venous cannula for blood collection was inserted into each ewe and kept patent with heparinized saline. Blood samples (3 ml) for the measurement of LH were collected at 10-min intervals for 12 h between 08.00 h and 20.00 h on the day of each experiment. After an initial 4-h control period, the first of two i.c.v. injections was made and a second given after a further 1.5 h. The order of the various injections was based on a cross-over design so that each ewe served as her own control and received each treatment. In some cases, the sampling period was extended to 13 h and a test of the response he pituitary gland to exogenous LHRH was made by injecting 425 pmol) ng) LHRH intravenously.

For the analysis of the LH data, the sampling period was split into three h divisions corresponding to times before, during and after the central ctions.

. Results

: concentration of LH in blood samples obtained every 10 min from riectomized control ewes injected with 50 µl sterile saline was pulsatile ɔughout the 12-h sampling period. During this time, the LH pulse ɹuency, pulse amplitude and mean LH concentration did not change ıificantly with time. The central administration of LHRH, however, ılted in a dose-related inhibition of LH secretion.

The 12-h LH profiles demonstrating the effects of injection of saline and two doses of LHRH into the third ventricle of an individual ewe are shown in Figure 4.1. We found no effect of i.c.v. saline on LH release (Figure 4.1a). However, when 2.1 pmol LHRH was injected i.c.v. (Figure 4.1b) there was a gradual reduction in the pulsatile nature of LH secretion which, in the particular ewe illustrated, gradually returned towards the end of the sampling period. The dose-related nature of this effect of central LHRH is also shown in this figure. Injection of 21 pmol LHRH (Figure 4.1c) completely inhibited the pulsatile release of LH, although the effect was not immediate. In an attempt to find out how long this suppression continued, the animals' sampling cannulae were left in place on one occasion and a brief (4-h) bleed undertaken the next day, starting at 09.00 h (i.e., 21 h after the first i.c.v. injection). In a few cases the pulsatile secretion of LH was still suppressed, whereas in other animals LH pulses had returned to the normal ovariectomized pattern.

The grouped effects of saline and LHRH administration on LH pulse frequency, pulse amplitude and mean LH levels in all six animals are shown in Figures 4.2a–c, 4.2d–f and 4.2g–i, respectively. The higher concentration (21 pmol) of LHRH suppressed pulse frequency significantly (Figure 4.2c) whereas the lower concentration (2.1 pmol) showed a much less consistent effect (Figure 4.2b). Both doses of LHRH, however, suppressed pulse amplitude in the third time period when compared with the first two (Figure 4.2e, f). Overall, both doses reduced mean LH levels, but not until the third time period (Figure 4.2h, i). From this figure, it might appear that the higher dose had an initial stimulatory effect; however, any such effect was not statistically significant and was largely a result of a wide inter-animal variation in pulse amplitude.

During the LHRH-induced inhibition of LH release, the pituitary gland responded to an i.v. bolus of 425 pmol LHRH with the release of a large pulse of LH. This is shown in Figure 4.3 for two individual ewes.

The effects of the LHRH antagonist on the inhibitory effect of central LHRH are shown for an individual ewe in Figure 4.4. Injection of 69 pmol LHRH antagonist (Figure 4.4a) had little or no effect on LH release, whereas 21 pmol LHRH (Figure 4.4b) inhibited LH secretion throughout the period of measurement. However, when the same amount of LHRH was injected into the third ventricle after the ewe had received a central injection of the LHRH antagonist 15 min earlier, the pattern of LH secretion was unaffected (Figure 4.4c).

Figure 4.1. Concentration of LH in 10-min plasma samples obtained over a 12-h sampling period in an individual ewe (No. 6E96) showing the dose-related inhibition of LH secretion by central LHRH. (a) effect of i.c.v. saline (50 µl). (b) effect of i.c.v. LHRH (2.1 pmol). (c) effect of i.c.v. LHRH (21 pmol). Injections were made into the third ventricle at arrows.

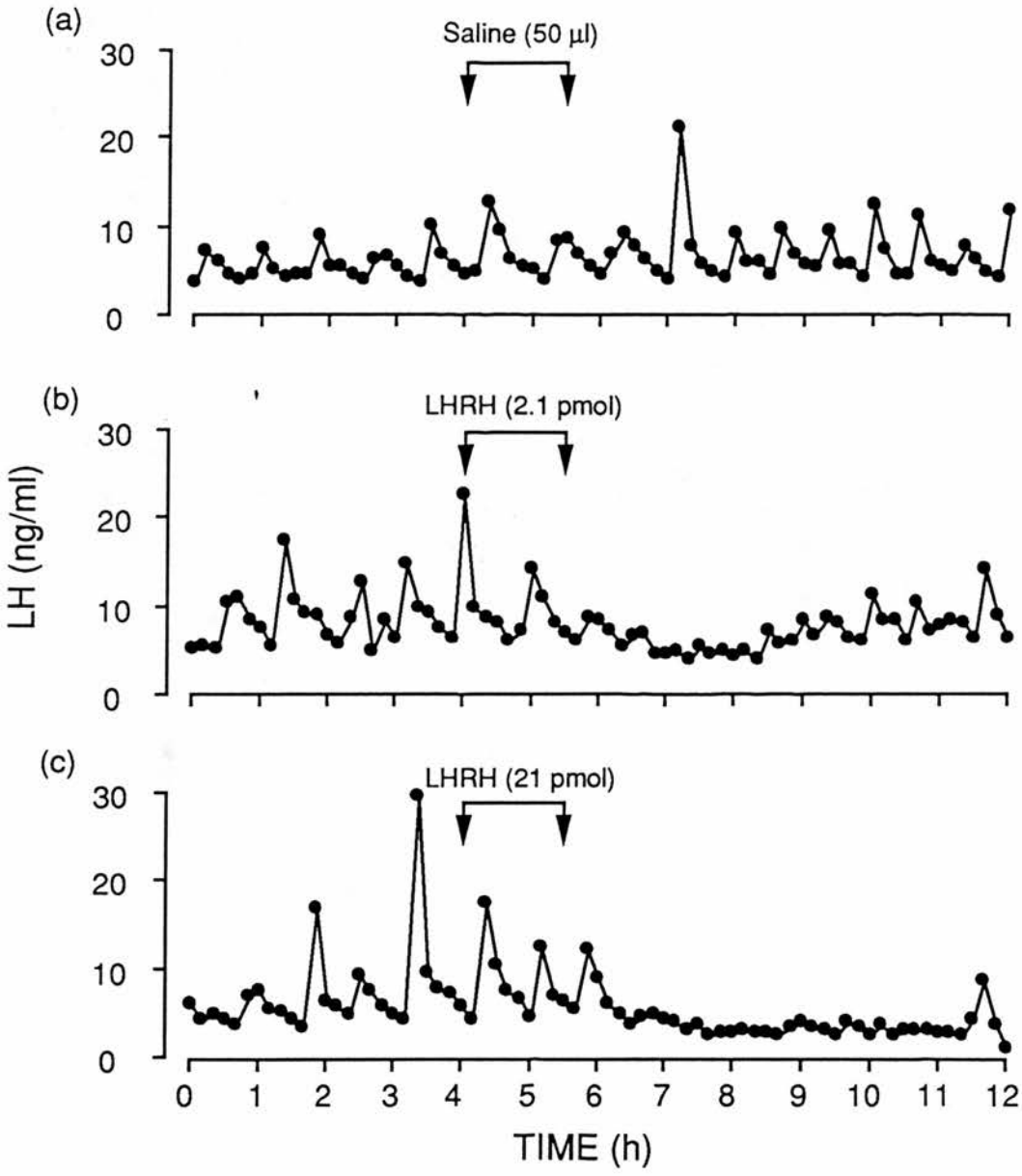

(a)
Saline (50 µl)
30
20
10
0
(b)
LHRH (2.1 pmol)
30
20
10
0
LH (ng/ml)
(c)
LHRH (21 pmol)
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
TIME (h)

Figure 4.2. Grouped data demonstrating the effect of 3 i.c.v. treatments (saline and 2 concentrations of LHRH) on 3 parameters of LH secretion, pre-, during- and post-injection. (a–c) LH pulse frequency (pulses per 3.5-h sampling period). (d–f) LH pulse amplitude. (g–i) mean LH levels. Values are expressed as the mean ± s.e.m. (n = 6). Levels of significance are indicated where differences exist.

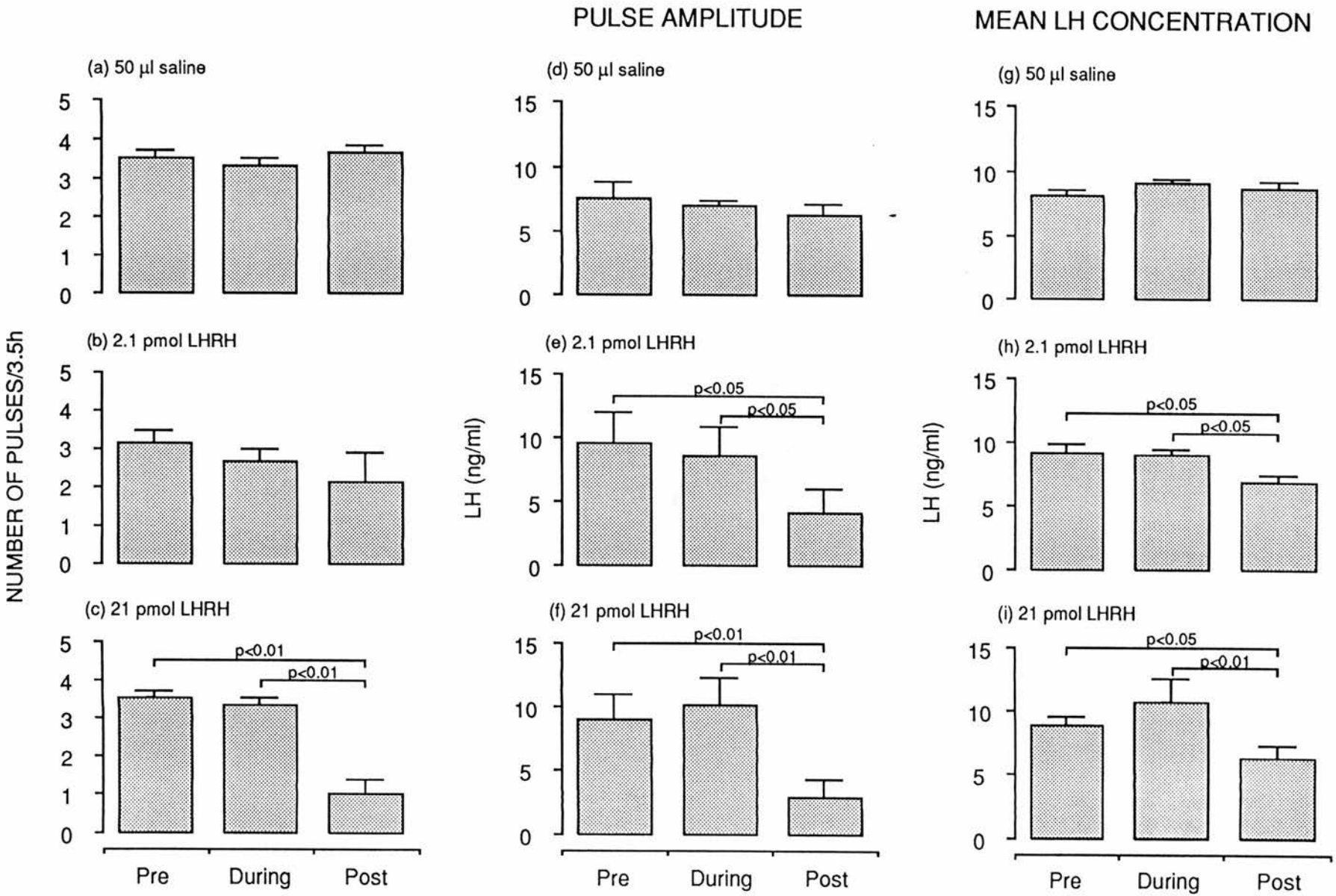

PULSE AMPLITUDE
MEAN LH CONCENTRATION
(a) 50 µl saline
(b) 2.1 pmol LHRH
(c) 21 pmol LHRH
(d) 50 µl saline
(e) 2.1 pmol LHRH
(f) 21 pmol LHRH
(g) 50 µl saline
(h) 2.1 pmol LHRH
(i) 21 pmol LHRH
NUMBER OF PULSES/3.5h
LH (ng/ml)
LH (ng/ml)
p<0.05
p<0.01
Pre
During
Post

Figure 4.3. Concentration of LH in 10-min plasma samples obtained over a 13-h sampling period in two individual ewes (Nos. 106 and 6DF7) showing the effect of a bolus i.v. injection of LHRH (425 pmol at arrow) on LH release during the period of suppression. Central injections of LHRH (21 pmol) were made into the third ventricle as previously at arrows.

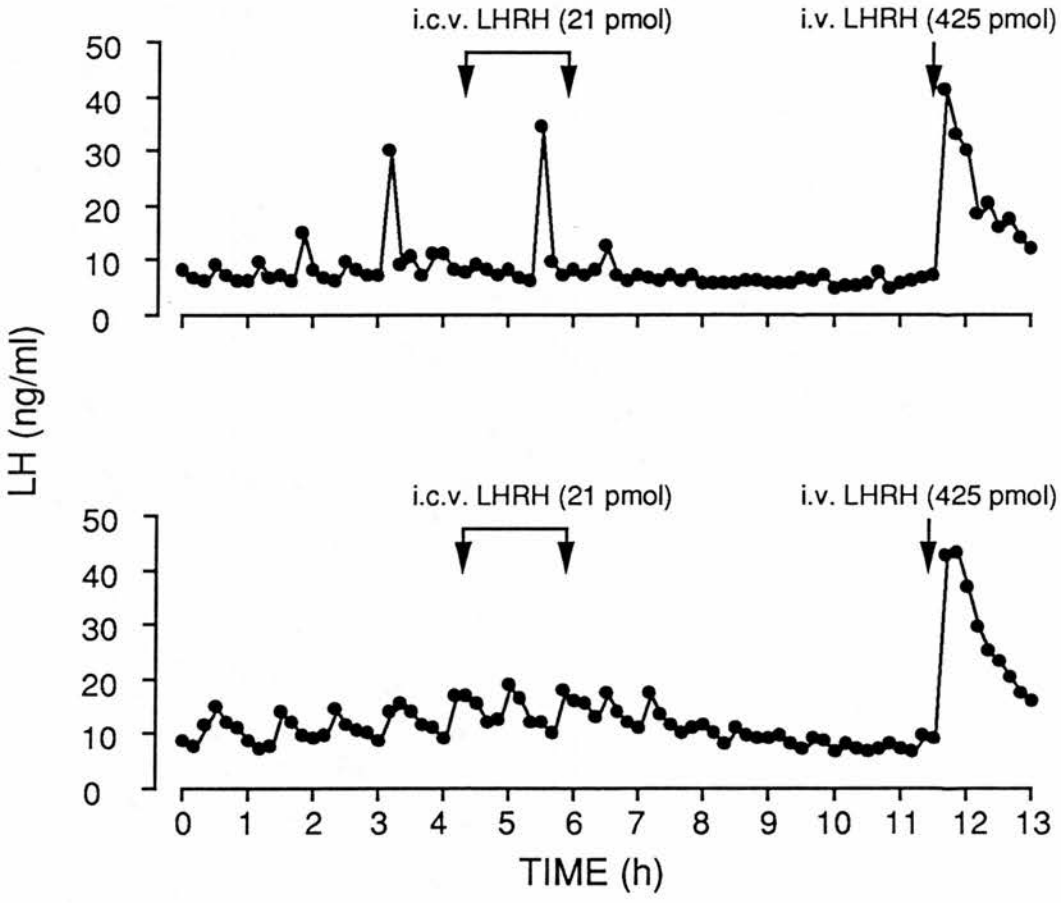

i.c.v. LHRH (21 pmol)
i.v. LHRH (425 pmol)
i.c.v. LHRH (21 pmol)
i.v. LHRH (425 pmol)
LH (ng/ml)
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
TIME (h)

Figure 4.4. Concentration of LH in 10-min plasma samples obtained over a 12-h sampling period in an individual ewe (No. 115) showing that pretreatment with the LHRH antagonist (injected i.c.v. 15 min before i.c.v. LHRH, twice) prevented the inhibitory effect of central LHRH. (a) effect of i.c.v. LHRH antagonist (69 pmol). (b) effect of i.c.v. LHRH (21 pmol). (c) effect of i.c.v. LHRH (21 pmol) in the presence of LHRH antagonist (69 pmol, i.c.v.). Injections were made into the third ventricle at arrows.

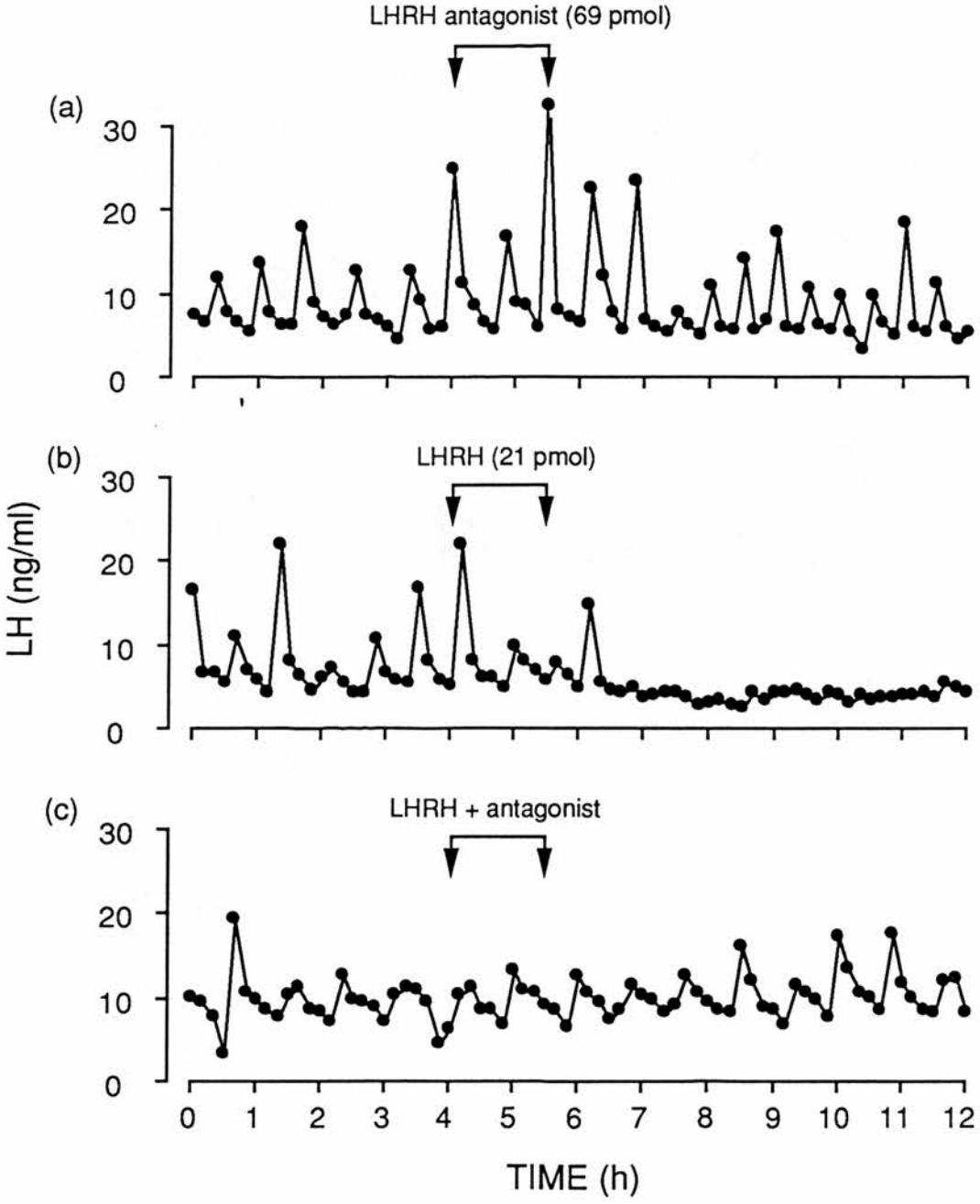

LHRH antagonist (69 pmol)
(a)
30
20
10
0
(b)
LHRH (21 pmol)
30
20
10
0
LH (ng/ml)
(c)
LHRH + antagonist
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
TIME (h)

Figure 4.5 shows the grouped effects of LHRH antagonist and LHRH plus antagonist on LH pulse frequency and mean LH levels for all six animals. Pretreatment with the LHRH antagonist blocked the ability of central LHRH to reduce pulse frequency (Figure 4.5, left) and pulse amplitude (data not shown). Likewise, the inhibitory effect of central LHRH on mean LH levels was blocked by prior treatment with the antagonist (Figure 4.5, right).

In contrast to its effect on LH, central injection of LHRH did not alter the pattern of FSH or prolactin secretion when compared with the control responses to saline (Figure 4.6).

In a preliminary experiment the LHRH agonist, Buserelin, was injected centrally. Somewhat surprisingly, we found that despite the action of LHRH itself, the analogue had no effect on LH pulse frequency, pulse amplitude or mean LH levels. The grouped effects of Buserelin on LH pulse frequency and mean levels are shown in Figure 4.7.

4.4. Discussion

Injection of LHRH into the third cerebral ventricle of the ovariectomized ewe caused a delayed (2–3 h) inhibition of LH secretion that was sustained for at least 6 h in most cases and possibly for up to 20 h or more. Pulse frequency and mean LH levels were reduced significantly following central LHRH administration when compared with the control responses aline. In addition, the pulses of LH that were evident were reduced siderably in magnitude. In contrast, plasma levels of FSH and lactin were unaffected by centrally injected LHRH. An absence of an ibitory effect of central LHRH on FSH secretion has been described viously in the rat (DePaolo *et al.* 1987) and is not unexpected in view of fact that the half-life of FSH in plasma is 2–3 h or more (Fry, Cahill, nmins *et al.* 1987). In addition, FSH secretion is normally unaffected by rt-term interruptions in the supply of LHRH to the pituitary gland ser & McNeilly, 1983). The unchanged prolactin levels suggest that the uced secretion of LH is not a consequence of hyperprolactinaemia hen-Becker, Selmanoff & Wise, 1986).

The inhibitory effect of central LHRH was dose-related in that the ıer dose always suppressed LH release whereas the lower dose exerted consistent effects, suppressing mean LH levels significantly but not ring pulse frequency. Both concentrations of LHRH suppressed pulse

Figure 4.5. Grouped data illustrating the effects of 2 treatments (antagonist alone and LHRH plus antagonist) on LH pulse frequency (left) and mean LH concentration (right). Doses are given in the figure. For effects of LHRH on LH pulse frequency and mean LH, see Figures 4.2a–c and 4.2g–i, respectively. Pretreatment with antagonist blocked the LHRH-induced suppression of LH secretion. Values are expressed as the mean ± s.e.m. (n = 6).

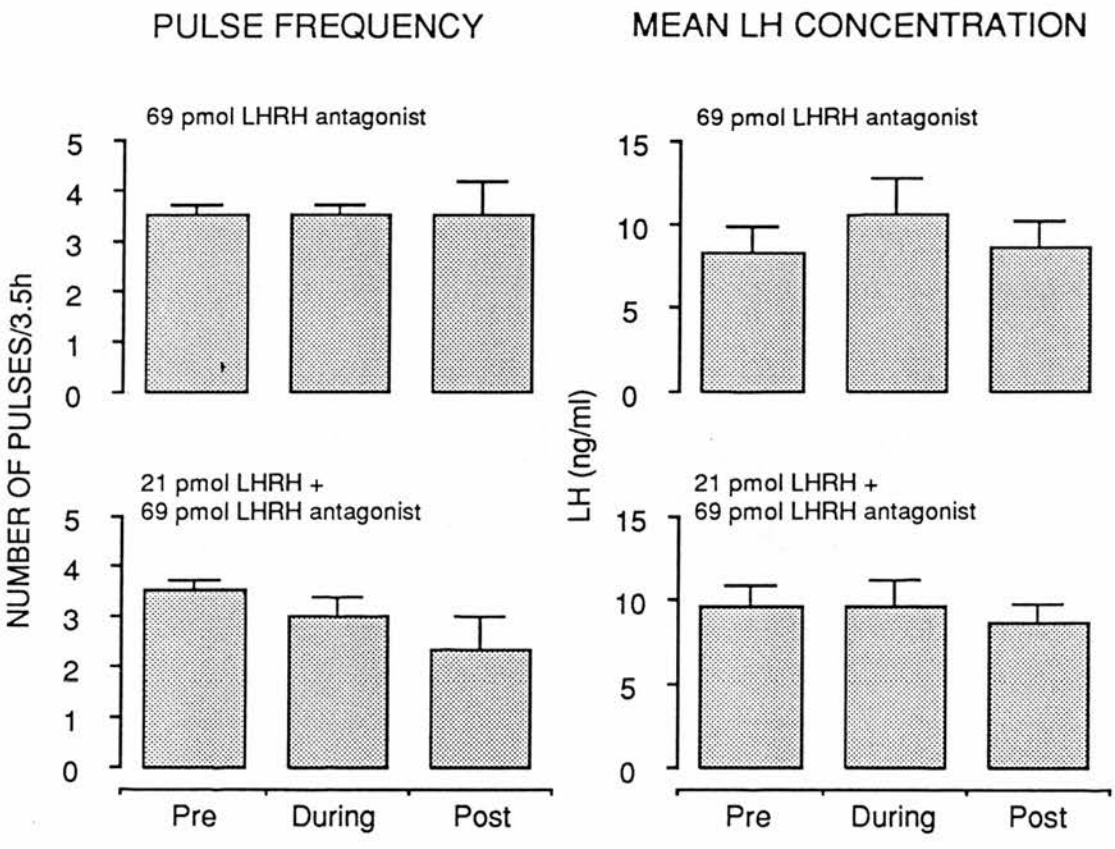

PULSE FREQUENCY
MEAN LH CONCENTRATION
69 pmol LHRH antagonist
21 pmol LHRH +
69 pmol LHRH antagonist
NUMBER OF PULSES/3.5h
LH (ng/ml)
0
1
2
3
4
5
0
5
10
15
Pre
During
Post

Figure 4.6. Effects of central saline (50 µl) or LHRH (21 pmol) on the mean hourly plasma levels of FSH (upper) and prolactin (lower). Central injection of LHRH had no effect on FSH or prolactin secretion. Values are expressed as the mean ± s.e.m. (n = 6).

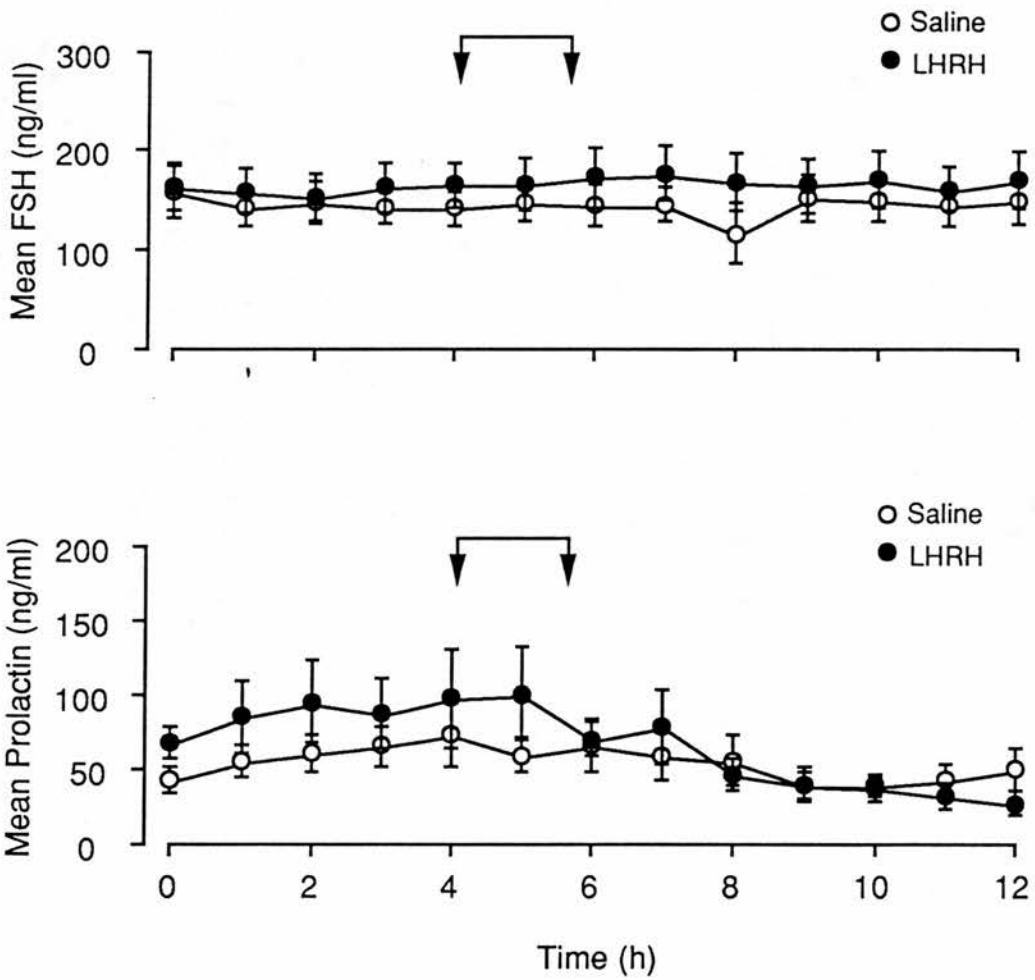
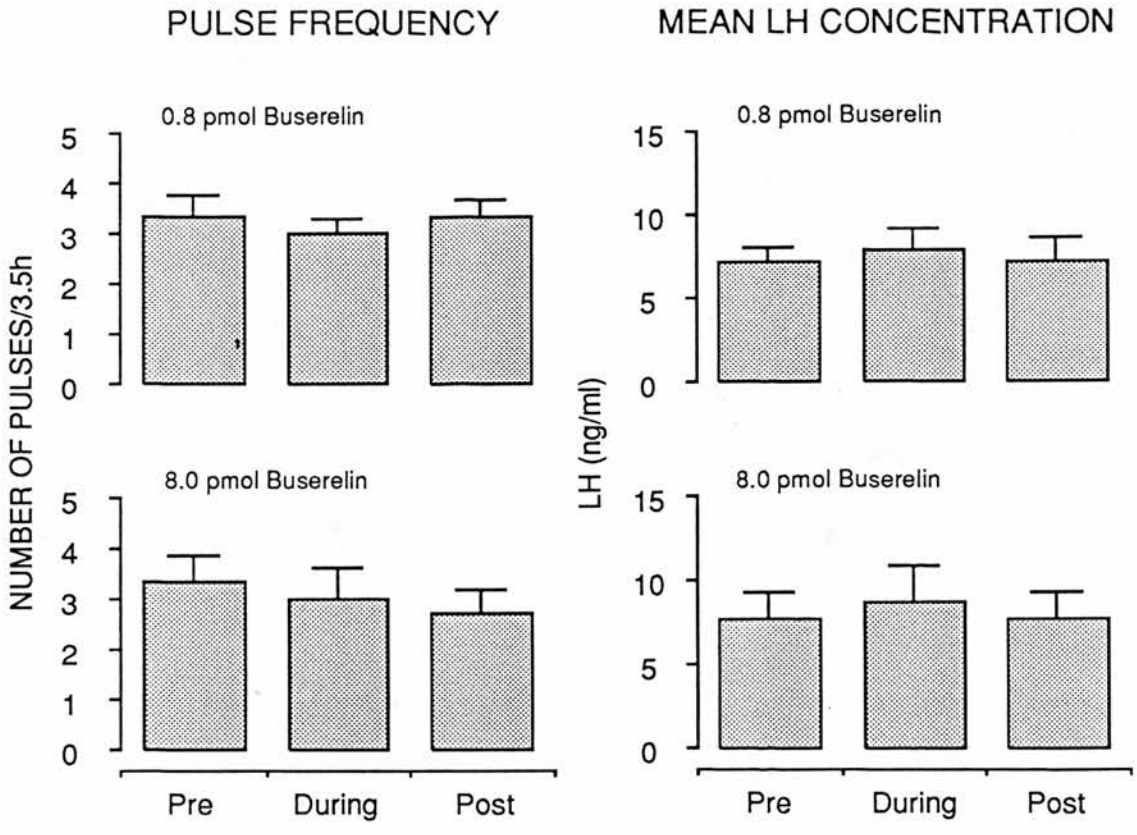

Saline
LHRH
Mean FSH (ng/ml)
300
200
100
0
Saline
LHRH
Mean Prolactin (ng/ml)
200
150
100
50
0
0
2
4
6
8
10
12
Time (h)

Figure 4.7. Grouped data showing the effects of i.c.v. LHRH agonist (Buserelin, 0.8 pmol and 8.0 pmol) on LH pulse frequency (left) and mean LH concentration (right). LHRH agonist had no effect on LH secretion. Compare effects of LHRH, shown in Figures 4.2a–c and 4.2g–i. Values expressed as the mean ± s.e.m. (n = 6).


PULSE FREQUENCY
MEAN LH CONCENTRATION
0.8 pmol Buserelin
8.0 pmol Buserelin
NUMBER OF PULSES/3.5h
LH (ng/ml)
0
1
2
3
4
5
0
5
10
15
Pre
During
Post

amplitude significantly. One question that arises is whether the decrease in pulse frequency is due to a slowing down of the LHRH pulse generator or whether a reduction in LHRH pulse amplitude is responsible. From the results of these experiments it is tempting to speculate that the latter may be the case since in ovariectomized hypothalamo-pituitary disconnected sheep, there is an inverse relationship between LHRH/LH pulse frequency and LH pulse amplitude (Clarke, Cummins, Findlay *et al.* 1984). Although fewer pulses were observed in the post LHRH period, those that were evident were reduced considerably in magnitude. This suggests that, in the sheep, the effect of central LHRH may be to suppress pulse amplitude (i.e., reduce LHRH release) rather than to slow down the actual frequency of the pulse generator. In support of this view, an LHRH agonist suppresses the release of LHRH *in vitro* (Bourguignon *et al.* 1987; Zanisi, Messi, Motta & Martini, 1987). However, in the rat, central injection of an LHRH agonist suppresses both the amplitude and frequency of LHRH pulses (Sarkar, 1987).

A common feature we observed was the delay in response following an i.c.v. injection of LHRH. The reason for this is unclear from the results of these experiments but it is highly possible that it may be due to the activation of another neuronal system, which is then responsible for the reduction in LHRH and therefore in LH. Indeed, the next two chapters describe experiments investigating two possible candidate systems. Alternatively, it may be due to the time required for centrally injected LHRH to reach active sites in the brain. This appears unlikely as the LHRH would presumably be inactivated by enzymes in the CSF and extracellular spaces. Similarly, the mechanisms underlying the sustained nature of the inhibition remain unclear. It might be argued that the exogenously administered LHRH caused a pituitary desensitization to endogenous LHRH. However, this is unlikely for two reasons. Firstly, there was no evidence of 'sensitization' which usually precedes a desensitization and secondly, bolus intravenous injection of LHRH still evoked a prompt and marked release of LH from the pituitary gland despite the lack of endogenous activity. These observations, coupled with the relatively low dose of LHRH required, suggest that the LHRH-induced suppression of LH secretion is a central phenomenon.

A similar inhibitory effect of central LHRH has been reported for the *in vivo* (Bedran de Castro *et al.* 1985; DePaolo *et al.* 1987; Sarkar, 1987). However, in the rat, the effect of LHRH was not so delayed nor so

sustained as that described here in the sheep. However, in both the rat and the sheep, the evidence implicates the central nervous system (CNS) rather than the pituitary gland in the response. In the rat, there is evidence from in-vitro studies that cell bodies (DePaolo *et al.* 1987) and nerve terminals (Valença *et al.* 1987) in the mediobasal hypothalamus are involved. However, it is impossible to determine from these experiments the relative contribution of each to the LHRH-induced inhibition of LH secretion in the ewe.

Although no studies have been undertaken to characterize LHRH receptors in the sheep brain, the fact that we have shown LHRH to exert central actions supports the hypothesis that they exist. Also, our demonstration that an LHRH antagonist is capable of preventing the LHRH-induced suppression of LH release strengthens the suggestion that the inhibitory effect of LHRH involves an interaction with LHRH receptors, rather than a non-specific action. This was achieved using a concentration of LHRH antagonist that did not affect any characteristic of LH secretion when administered alone. A similar reversal of inhibitory action (induced by an LHRH agonist) has been described using another LHRH antagonist ([Ac-*D*-Ala^1, *D*-Phe^2, *D*-Trp^{3,6}]LHRH) (Sarkar, 1987).

In an attempt to determine the functional significance of endogenous LHRH in regulating pulse generator activity, a 5-fold higher concentration of the LHRH antagonist (345 pmol) was injected into the third ventricle. However, at this high dose, the antagonist exerted inhibitory actions, presumably by travelling in the portal system to the pituitary where it

:ked LHRH receptors. Therefore, the current experiments produced no
lence to suggest that endogenous LHRH is involved in the
siological control of LHRH secretion. However, it is possible that
rete microinjection of relatively low doses of LHRH antagonist into
:tionally important sites in the sheep hypothalamus may reveal a role
endogenous LHRH in the control of the LHRH pulse generator. In
port of this, there is evidence from studies using LHRH antagonists *in*
) that endogenous LHRH exerts a tonic inhibitory influence on LHRH
etion in the rat (Valença *et al.* 1987).

The experiments described in this chapter were carried out in long-
ı ovariectomized ewes and so the inhibitory effect of central LHRH
we observed occurred in the absence of ovarian steroids. The
;atility of LHRH secretion under these conditions is nearly maximal
so this provides an explanation of why we were unable to show an

effect of the antagonist alone. Since oestradiol and progesterone exert such potent modulatory actions on the LHRH pulse generator (see Chapter 2), the question of the precise physiological significance of LHRH feedback remains open. It would be of great interest to determine the effects of central administration of LHRH antagonist during the luteal phase, for instance, as it seems highly probable that it would be possible to demonstrate a role for endogenous LHRH under these conditions.

In conclusion, the work described in this chapter indicates that in the sheep, administration of LHRH into the brain exerts a dose-related and receptor-mediated inhibition of pulse generator activity. This supports the hypothesis that central LHRH is involved in a system to regulate its own release. However, the delayed and sustained nature of the inhibitory effect suggests strongly that another neural or endocrine system is involved in LHRH autoregulation. Experiments designed to investigate two potential mechanisms whereby this may be achieved are described in the following two chapters.

5. Effect of the opiate antagonist naloxone on the LHRH-induced inhibition of LH secretion

5.1. Introduction

In the preceding chapter, evidence was presented to support the hypothesis that an autoregulatory mechanism exists in the control of central LHRH in the sheep. Centrally administered LHRH was observed to cause a marked inhibition of LH secretion, in agreement with the findings of Bedràn de Castro *et al.* (1985) and DePaolo *et al.* (1987) in the rat. As discussed previously, a reduction in the sensitivity of the pituitary gland to endogenous LHRH is unlikely to account for these findings. The inhibition of plasma LH is more likely to be due to an inhibition of LHRH release from LHRH nerve terminals (Bourguignon *et al.* 1987; DePaolo *et al.* 1987; Sarkar, 1987; Valença *et al.* 1987; Zanisi *et al.* 1987).

Bedran de Castro *et al.* (1985) and DePaolo *et al.* (1987) observed a transient reduction in LH secretion within minutes of injection of LHRH into the third ventricle of the rat. In contrast, we found the onset of the suppressive effects of central LHRH to be delayed by 2–3 h and the bition itself to last for at least 6 h and possibly for up to 20 h after ction. This suggests that, in the sheep, the activation of another ral or endocrine system may ultimately be responsible for the action in LHRH and therefore in LH. Several possible candidate hanisms could be responsible. Firstly, a raised plasma prolactin centration might mediate the response. Secondly, activation of one of 'classical' neurotransmitter pathways such as those involving inobutyric acid, noradrenaline, or the endogenous opioid peptides be responsible. Thirdly, our findings could also be accounted for by vation of another neuroendocrine system such as the hypothalamo-itary-adrenal axis.

The first putative mechanism can be discounted. Although erprolactinaemia has been shown to reduce pulsatile LH secretion nen-Becker *et al.* 1986) and to reduce pituitary portal levels of LHRH kar & Yen, 1985), we found no evidence of any alterations in plasma

prolactin (see Chapter 4).

We decided to investigate further the possible actions of the endogenous opioid peptides and the hypothalamo-pituitary-adrenal axis in the autoregulation of LHRH. This chapter describes our experiments on the role of the EOPs. Our studies investigating the role of the hypothalamo-pituitary-adrenal axis are described in Chapter 6.

Endogenous opioid peptides have been implicated in the inhibitory control of LHRH release in a number of species (Cicero, Schainker & Meyer, 1979; Kinoshita, Nakai, Katakami *et al.* 1980; Schultz, Wilhelm, Pirke *et al.* 1981; Sarkar & Yen, 1985; Wiesner, Koenig, Krulich & Moss, 1985; Orstead & Spies, 1987), including the sheep (Brooks, Lamming & Haynes, 1986; Horton, Francis & Clarke, 1988). In particular, intracerebroventricular (i.c.v.) injection of naloxone or of anti-β-endorphin antiserum augments LH release in the ewe (Weesner & Malven, 1988). Since endogenous opioid peptides mediate the inhibitory effects on LHRH/LH release of a number of neuropeptides including corticotrophin-releasing hormone (CRH) (Gindoff & Ferin, 1987; Almeida, Nikolarakis & Herz, 1988; Nikolarakis, Almeida & Herz, 1988; Nikolarakis, Almeida, Sirinathsinghji & Herz, 1988) and atrial natriuretic factor (ANF) (Samson, Aguila & Bianchi, 1988), it is conceivable that activation of endogenous opioid pathways, e.g. those containing β-endorphin, may participate in the prolonged inhibition of LH secretion described in the preceding chapter.

The following studies were therefore undertaken in ovariectomized ewes to determine whether, like their involvement in the suppressive effect of CRH and ANF, endogenous opioid peptides are involved in mediating the delayed and sustained inhibitory effects of central LHRH on episodic LH secretion. In addition, we were able to investigate the presence of an opioid-mediated tonic inhibitory influence on LHRH/LH release in long-term ovariectomized ewes by administering the opioid antagonist naloxone on its own.

Experimental design

Scottish Blackface ewes were housed in individual pens in the University of Edinburgh Marshall Building, near Edinburgh, under artificial lighting conditions (14 h light; 10 h dark) from January to March 1. The sheep were fed concentrated pellets once a day, and hay and water were available *ad libitum*.

Two to three months before experimentation, the animals were ovariectomized and implanted with a stainless steel cannula directed towards the third ventricle as described in Chapter 3.

To study whether endogenous opioids are involved in mediating the inhibition of LH secretion described in the previous chapter, we repeated the i.c.v. injections of LHRH at the higher dose (21 pmol) with or without a concomitant intravenous administration of naloxone. The animals also received control injections of i.c.v. saline and i.v. naloxone alone. 25 mg naloxone (see Section 3.4) was injected four times intravenously at intervals of 1.5 h. Injections of naloxone were made down the sampling cannula immediately after a blood sample was taken and before the line was flushed with heparinized saline.

On the day before each experiment began, a jugular venous cannula was inserted into each ewe and kept patent with heparinized saline. Blood samples (3 ml) for the measurement of LH were collected at 10-min intervals for 11 h between 08.00 h and 19.00 h on the day of each experiment. After an initial 4-h control period, the first of two i.c.v. injections was made. The second injection was made after a further 1.5 h. Naloxone (4 x 25 mg) was injected intravenously at 4, 5.5, 7 and 8.5 h into the sampling period. Each ewe received each treatment (i.c.v. saline, i.c.v. LHRH, i.v. naloxone, i.c.v. LHRH plus i.v. naloxone) in random order and so acted as her own control.

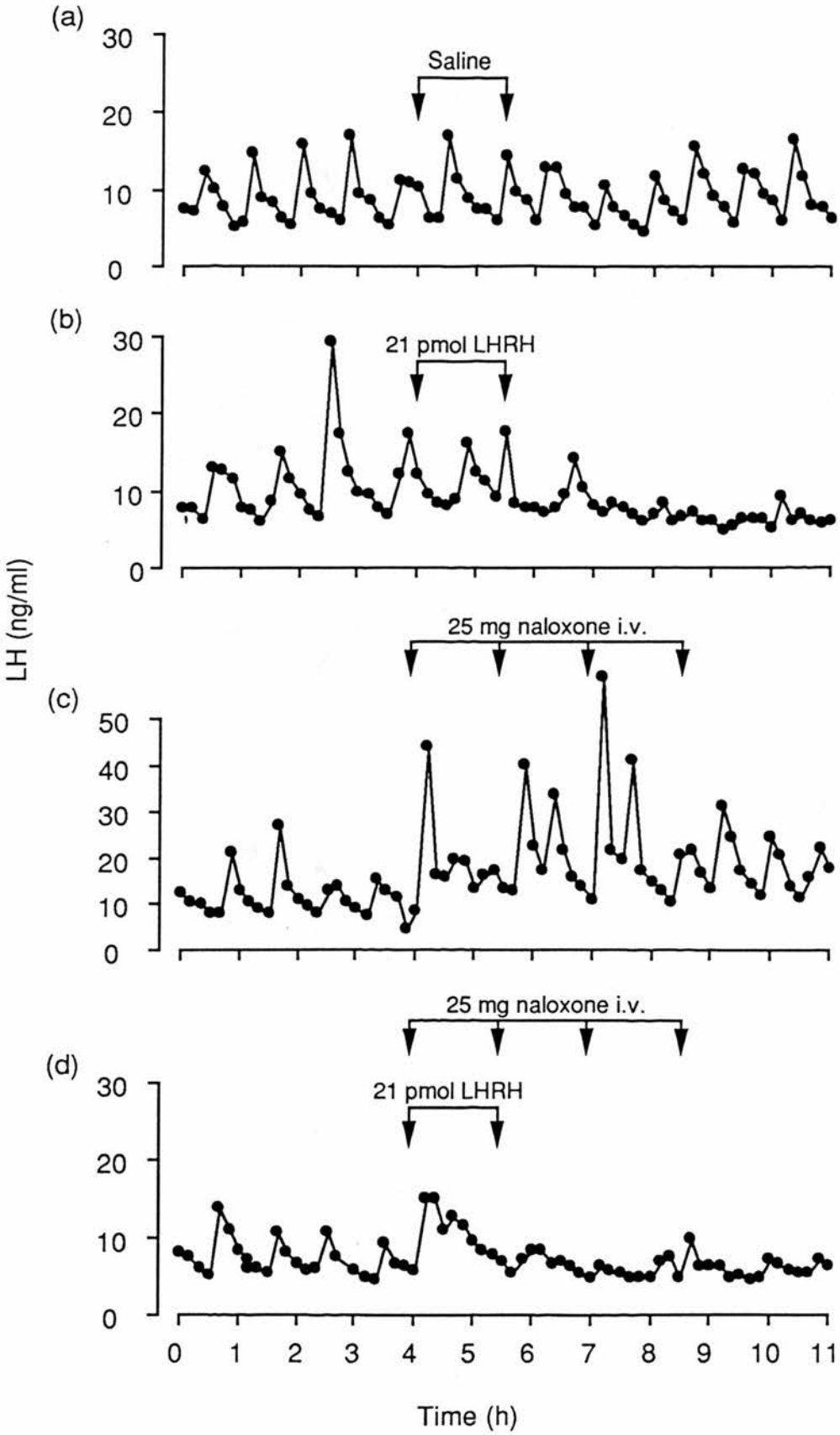
For the analysis of the LH data, the sampling period was divided into ee equal 3.5-h windows corresponding to times before, during and after central injections.

. Results

e concentration of LH in blood samples obtained every 10 min from g-term ovariectomized control ewes injected with 50 µl saline i.c.v. was satile throughout the 11-h sampling period.

The 11-h pulse profiles obtained from an individual ewe, showing the ects of the four treatments on pulsatile LH secretion, are shown in ure 5.1. Injection of saline into the third cerebral ventricle (Figure 5.1a) not influence the normal pulsatile pattern of LH secretion. However, en 21 pmol of LHRH was injected i.c.v. (Figure 5.1b) the normal profile LH secretion was altered. There was a gradual reduction in the pulsatile ase of LH which, 7 h after injection, was almost completely inhibited

Figure 5.1. Concentration of LH in 10-min plasma samples obtained over an 11-h sampling period in an individual ewe showing the effects of four treatments. (a) effect of i.c.v. saline (50 μl). (b) effect of i.c.v. LHRH (21 pmol). (c) effect of i.v. naloxone (4 x 25 mg) (note change of scale). (d) effect of i.c.v. LHRH (21 pmol) plus i.v. naloxone (4 x 25 mg). Times of injections are indicated by arrows.


(a)
Saline
(b)
21 pmol LHRH
25 mg naloxone i.v.
(c)
25 mg naloxone i.v.
(d)
21 pmol LHRH
LH (ng/ml)
Time (h)

in the ewe shown. Despite the stimulatory effects of naloxone alone (Figure 5.1c), the sustained inhibition of LHRH/LH produced by central LHRH persisted in the presence of the opioid antagonist (Figure 5.1d).

The grouped effects of the treatments on two parameters of LH secretion, mean concentration and pulse frequency, are shown for all five animals in Figures 5.2 and 5.3 respectively. Injection of 50 µl saline i.c.v. did not affect mean LH levels (Figure 5.2a) or LH pulse frequency (Figure 5.3a). Central LHRH (21 pmol) reduced mean plasma LH concentrations significantly compared with the time periods before and during i.c.v. injection (Figure 5.2b), confirming our previous findings (Chapter 4). Intravenous administration of naloxone (4 x 25 mg) resulted in a significant increase in LH release which was evident throughout the sampling period (Figure 5.2c). However, in the presence of naloxone, third ventricular'injection of LHRH still caused a significant reduction in mean LH concentration (Figure 5.2d). The effects of saline, LHRH and LHRH plus naloxone on LH pulse frequency are shown in Figure 5.3. Third ventricular administration of LHRH caused a significant reduction in pulse frequency (Figure 5.3b) which was not reversed by naloxone (Figure 5.3c). The large increase in mean LH concentration after i.v. naloxone was due to an increase in pulse frequency in four out of five ewes ($p = 0.085$) and a significant increase ($p < 0.05$) in pulse amplitude (Figure 5.4).

Discussion

ction of LHRH into the third cerebral ventricle of the ovariectomized resulted in a delayed but sustained inhibition of LHRH/LH secretion. se frequency and mean LH levels were significantly reduced. In ition, the pulses of LH that were recorded were reduced considerably in nitude. This confirms the findings of the previous chapter. The data demonstrate that concomitant infusion of naloxone has no effect on response to LHRH. Therefore, in contrast to their role in mediating inhibitory actions of CRH and ANF on LHRH release, endogenous oid peptides are unlikely to be involved in the LHRH-induced bition of LHRH/LH secretion.

The concept of autoregulation (or 'ultrashort'-loop feedback as cribed in Section 5.1) has been used to describe the inhibitory effect of ral LHRH on LHRH/LH secretion in the rat (Bedran de Castro *et al.*

Figure 5.2. Grouped data showing the effects of 4 treatments on mean LH concentrations in the time periods before, during and after i.c.v. injection. (a) i.c.v. saline (50 μl). (b) i.c.v. LHRH (21 pmol). (c) i.v. naloxone (4 x 25 mg) (note change of scale). (d) i.c.v. LHRH plus i.v. naloxone. Values are expressed as the mean ± s.e.m. (n = 5).

MEAN LH CONCENTRATION

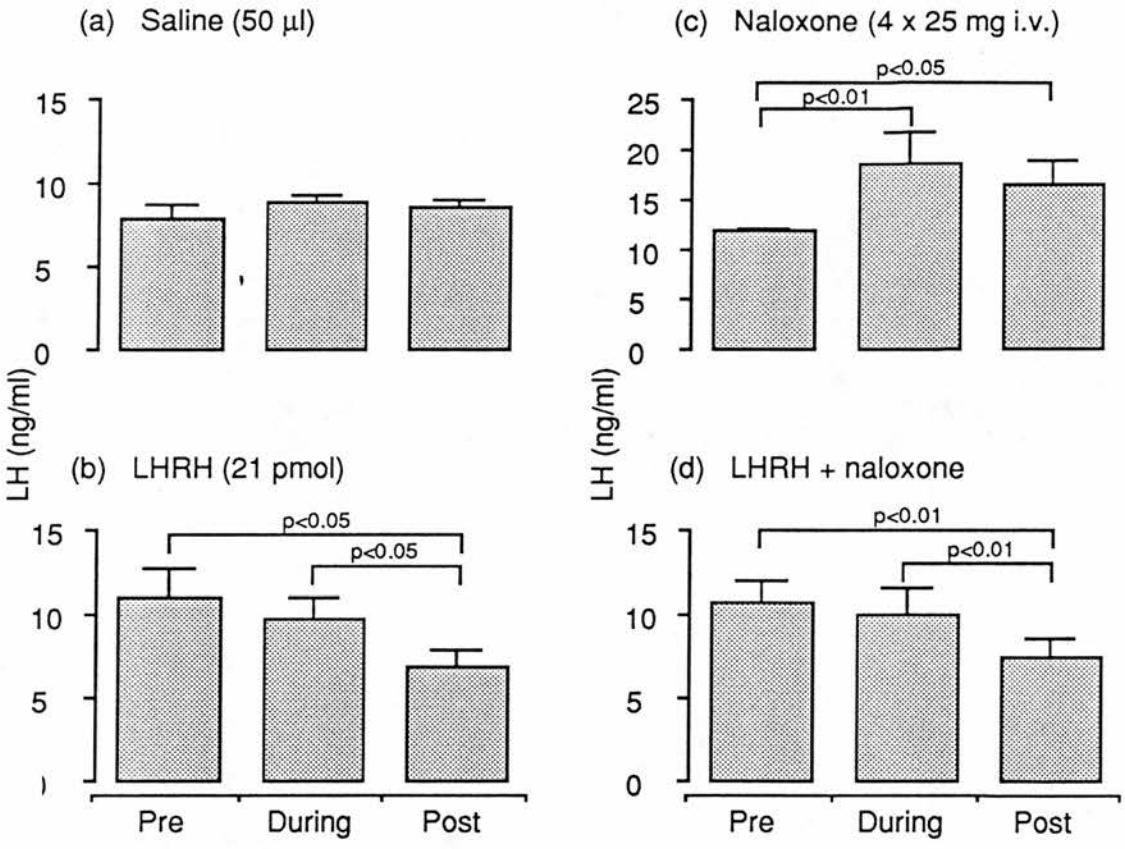


Figure 5.3. Grouped data showing the effects of 3 treatments on LH pulse frequency before, during and after i.c.v. injection. (a) i.c.v. saline (50 µl). (b) i.c.v. LHRH (21 pmol). (c) i.c.v. LHRH plus i.v. naloxone. Values are expressed as the mean ± s.e.m. (n = 5).

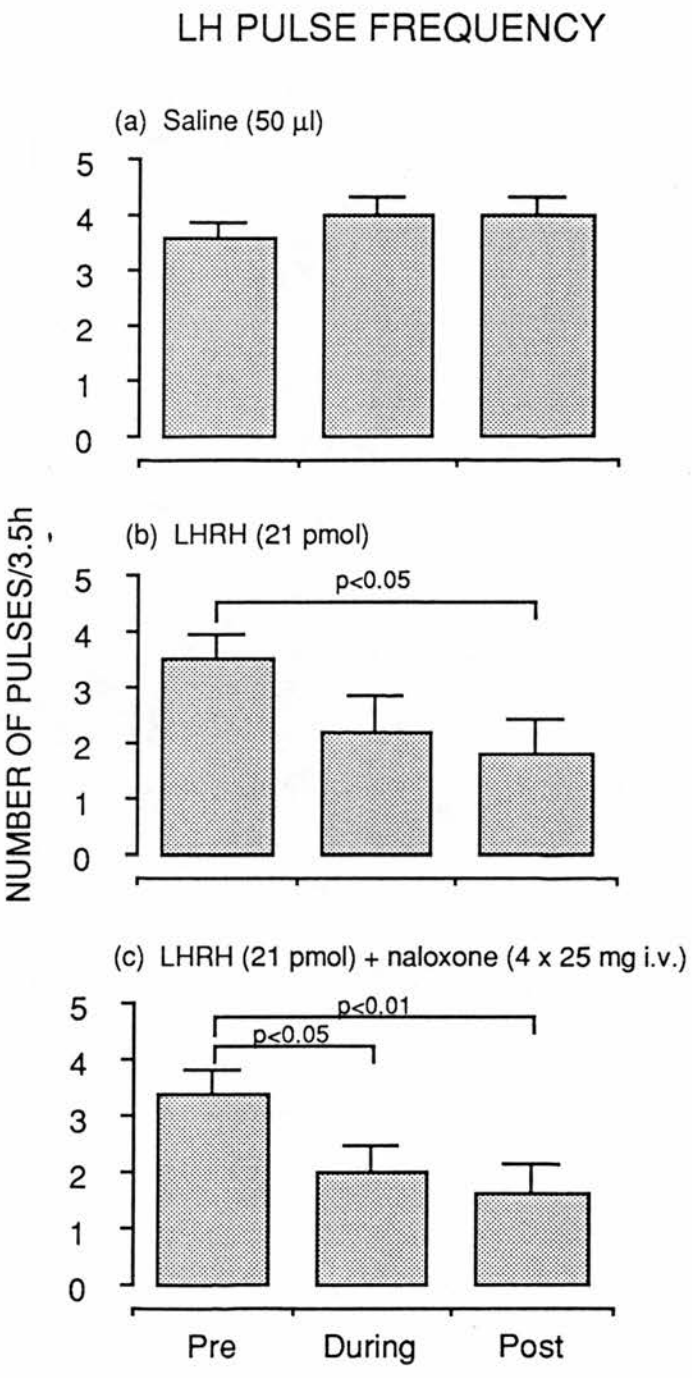
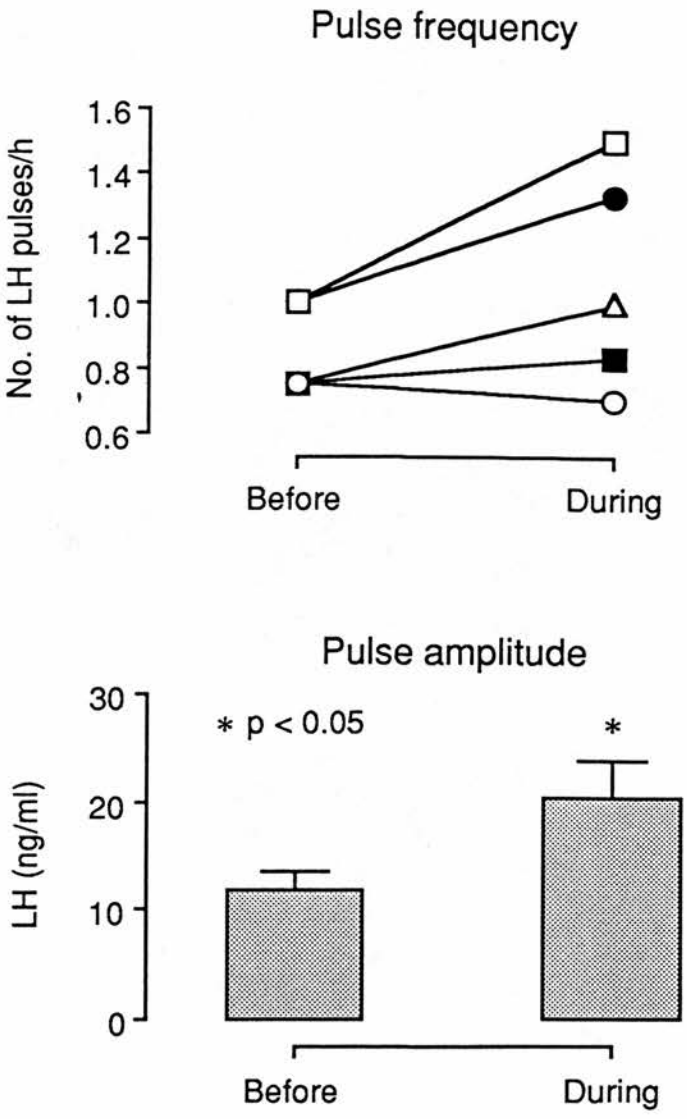

LH PULSE FREQUENCY
(a) Saline (50 µl)
(b) LHRH (21 pmol)
p<0.05
(c) LHRH (21 pmol) + naloxone (4 x 25 mg i.v.)
p<0.01
p<0.05
NUMBER OF PULSES/3.5h
0
1
2
3
4
5
Pre
During
Post

Figure 5.4. Effect of intravenous injection of naloxone (4 x 25 mg) on LH pulse frequency and pulse amplitude for all 5 ewes in the 3.5 h periods before and during treatment. Individual responses are shown for pulse frequency ($p = 0.085$) and the mean ± s.e.m. ($n = 5$) for pulse amplitude.


Pulse frequency
No. of LH pulses/h
1.6
1.4
1.2
1.0
0.8
0.6
Before
During
Pulse amplitude
LH (ng/ml)
30
20
10
0
* p < 0.05
*
Before
During

1985). In this species, the inhibitory action of central LHRH is immediate, short-lived and occurs *in vitro* (Bourguignon *et al.* 1987; DePaolo *et al.* 1987; Zanisi *et al.* 1987). In the rat, there is morphological evidence for such a feedback system as LHRH neuronal terminals have been observed to synapse on LHRH cell bodies (Leranth *et al.* 1985; Pelletier, 1987). If a similar process of autoregulation were occurring in the sheep, it might be expected that the inhibitory response would be observed sooner and be of a shorter duration. The mechanisms responsible for the actions of LHRH in the two species may, however, be quite different. Although the possibility of a direct action of LHRH remains, the injected LHRH would presumably be inactivated by enzymes in the cerebrospinal fluid (CSF) or brain extracellular spaces within the period of inhibition as discussed in the previous chapter.

Interestingly,'the LHRH-induced inhibition persisted during naloxone administration whereas, when the same animals were treated with naloxone alone, there was a marked stimulation of LHRH/LH release. These data suggest that endogenous opioid peptides that may be antagonized by naloxone are unlikely to mediate the inhibitory effect of LHRH in the ewe and secondly, that the central inhibitory effect of LHRH blocks the stimulatory effect of naloxone.

An alternative explanation for the delayed response may be that the inhibitory effect of central LHRH is in fact very rapid but masked by a simultaneous, but short-lived, augmentation of LHRH/LH release. In

port of this proposal is the following. The inhibitory effect of central RH has a long latency, but the stimulatory effect of naloxone is nediate. However, when naloxone and LHRH are administered ether naloxone has no stimulatory effect. This could be taken to imply , in fact, there is an early onset of the LHRH effects, comparable with time course of the effects of naloxone. From the results, there is no clusive evidence to determine whether or not this is the case.

t might be argued that an insufficient dose of naloxone was used to vent the actions of all endogenous opioids since this opioid blocker is a ferential μ-receptor antagonist with a much lower affinity for the δ- κ-receptors. The relative binding of naloxone at the μ-, δ- and tes is 0.85, 0.06 and 0.09, respectively (Paterson, Robson & terlitz, 1983). However, since injection of naloxone alone exerted cts on LHRH/LH release it seems reasonable to assume that an quate amount of antagonist was present to influence opioid/LHRH

regulatory systems.

In this experiment, the mean levels of LH increased following naloxone treatment as a consequence of an increase in pulse frequency (in four out of five animals) ($p = 0.085$) and a significant increase in pulse amplitude ($p < 0.05$). The increase in LH pulse frequency suggests a hypothalamic (i.e., on LHRH release) rather than a pituitary site of action for naloxone. In support of this, naloxone implants placed in the medial preoptic area and median eminence arcuate region stimulate LH release in rats (Kalra, 1981) as does intrahypothalamic injection of anti-β-endorphin antiserum or naloxone in sheep (Weesner & Malven, 1988). Since naloxone crosses the blood brain barrier and is detectable in significant amounts in the brain following intravenous administration (Berkowitz, Ngai, Hempstead & Spector, 1975; Berkowitz, 1976), it is likely that the effects of naloxone on LH release that we observed are mediated at the hypothalamic level.

The results of the experiment in this chapter also demonstrate that in the long-term ovariectomized ewe (i.e., 2–3 months), endogenous opioid peptides exert a tonic inhibitory influence on LHRH/LH secretion. This observation is in contrast to other reports where naloxone stimulated LH release in ovariectomized ewes only if they had been implanted with progesterone at the time of gonadectomy (Brooks, Haynes, Yang & Lamming, 1986; Trout & Malven, 1984). However, it is in agreement with other studies reporting the stimulatory effect of naloxone (Schillo, Kuehl ° ackson, 1985) and another opioid antagonist, WIN-44441-3, (Yang, nes, Lamming & Brooks, 1988) on LHRH/LH release in ovarimized ewes. Evidence obtained from other species also suggests that oxone-stimulated LHRH/LH release is not diminished following iectomy (Orstead & Spies, 1987; Karahalios & Levine, 1988), although adal steroids do exert a major influence on the effects of naloxone and r opioid antagonists on LH secretion (Haynes, Lamming, Yang *et al.*).

n conclusion, the results of this experiment indicate that central RH inhibits LHRH pulse generator activity by a mechanism that ably does not involve opioid peptides. However, the hypothesis that ther neuroendocrine mechanism is responsible for mediating the bition still remains. This is addressed further in the next chapter, in ch experiments to investigate the possible role of the hypothalamoitary-adrenal axis are described.

6. Involvement of the hypothalamo-pituitary-adrenal axis in the suppression of LH secretion by centrally-administered LHRH

6.1. Introduction

The experiments in this chapter follow on from the results of the previous two. In Chapter 4, experiments revealing an autoregulatory mechanism for LHRH in the sheep were described. Central administration of LHRH resulted in a suppression of LH secretion that lasted for at least 6 h after an apparently delayed onset. These two features differed from previous studies in the rat, where the effects on LH secretion were immediate and transient, and prompted us to investigate LHRH self-regulation further. Several putative mechanisms were suggested in the previous chapter (Section 5.1) and one of these, the endogenous opioid peptide system, was investigated. Our experiments indicated that the opioid peptides were probably not involved in the inhibition of LHRH pulse generator activity by central LHRH.

The experiments described in this chapter address the hypothesis that central inhibition of LHRH secretion involves some aspect of the ɔthalamo-pituitary-adrenal axis, which is activated under conditions hysical and environmental stress. There is clear evidence for a role for ı of the components of this axis in the suppression of LHRH/LH ɛtion. In the ovariectomized rhesus monkey, intravenous infusion of I (Gindoff & Ferin, 1987; Olster & Ferin, 1987) although not of ACTH o & Ferin, 1988) suppresses both pulsatile LH and FSH release. ıcerebroventricular injection of CRH induces a rapid decline in LH not FSH) secretion in the ovariectomized rat and abolishes the pro-rous LH surge in 50% of intact rats (Rivier & Vale, 1984). adotrophin secretion is also suppressed by long-term administration ɔrtisol in castrate male rhesus monkeys (Dubey & Plant, 1985), but is ɔred in response to intravenous infusion of pulsatile LHRH, implying cortisol acts at a suprapituitary level to interrupt hypothalamic LHRH

release. Moreover, cortisol, implanted into the mediobasal hypothalamus, prevents the onset of puberty (Smith, Johnson, Weick *et al.* 1971) as do ACTH and adrenalectomy (giving rise to increased endogenous ACTH levels) (MacFarland & Mann, 1977). Furthermore, both acute and chronic glucocorticoid treatment in the rat suppresses plasma LH and both the pre-ovulatory and the oestradiol-induced LH surge (Baldwin & Sawyer, 1974; Baldwin, 1979).

Thus, the inhibition of LH secretion observed following central administration of LHRH may be due to activation of one or other components of the hypothalamo-pituitary-adrenal axis. We therefore investigated this hypothesis by determining the effect of injection of LHRH into the third ventricle on the concentrations of cortisol in the plasma samples we had taken in Chapter 4. Unfortunately, we were unable to measure ACTH in these samples as they had been thawed and re-frozen several times for the estimation of LH, FSH and prolactin. ACTH bio- and immunoactivity is destroyed by such treatment, so we had to use plasma levels of cortisol as an index of the activity of this axis. In addition, we looked at the effect of an LHRH antagonist on the cortisol response and, in order to investigate the possible role that cortisol may play in the suppression of LH by central LHRH, we attempted to determine the effect of intravenous administration of cortisol on LHRH/LH secretion.

Experimental design

experiments described in this chapter are of two types. Firstly, central ɛriments to determine the effect of i.c.v. injection of LHRH and an ʁH antagonist on plasma cortisol concentrations; and secondly, pheral experiments to determine whether LHRH/LH secretion is ːted by intravenous injections or infusions of cortisol.

. Intracerebroventricular experiments

ːisol concentrations after central treatment with both doses of LHRH the LHRH antagonist, alone and together, were measured in the ma of five of the six Scottish Blackface ewes described in Chapter 4. sixth ewe, although included in the analysis of the LH data reported hat chapter, did not respond to any of the treatments. When we

measured this animal's cortisol levels after central administration of the higher dose of LHRH, there was no effect on cortisol levels either, again in contrast to the responses of the other five animals. Although the LH responses were sufficiently robust to accommodate a non-responding animal, we did not feel that anything further would be achieved by extracting and assaying this ewe's samples for cortisol for all the treatments. Cortisol concentrations are therefore presented here for the five responding animals only.

As described earlier, the sheep were housed in individual pens under artificial lighting conditions (14 h light; 10 h dark) in the University of Edinburgh Marshall Building, near Edinburgh, between November 1987 and January 1988. They were fed concentrated pellets each morning and had unlimited access to hay and water.

Three to four weeks before experimentation, the animals were ovariectomized and implanted with a stainless steel cannula directed towards the third cerebral ventricle. The animals were prepared for blood collection as described previously. Central injections of LHRH with or without antagonist and of antagonist alone were made into the third ventricle at 4 and 5.5 h into the 12-h sampling period (for details of doses, see Section 4.2; details of drugs are given in Section 3.4). All animals also received a control injection of 50 µl saline as described previously.

Plasma cortisol concentrations were measured by radioimmunoassay (see Section 3.5.4) in half-hourly blood samples and in 10-min samples for [illegible] after each i.c.v. injection.

. Cortisol injections

[illegible]he first experiment to determine whether intravenous cortisol [illegible]inistration could affect the secretion of LHRH and LH, we injected [illegible]isol intravenously at half-hourly intervals in a group of six [illegible]iectomized ewes. Four to eight months prior to experimentation, the [illegible]nals were ovariectomized as described previously. During the [illegible]riment, in July 1988, the animals were housed in approved restraint [illegible]s in the University of Edinburgh Marshall Building (16 h light; 8 h [illegible]) and received feed and water as previously.

[illegible])n the day before experimentation, bilateral jugular venous cannulae, [illegible] patent with heparinized saline, were inserted into each ewe. On the [illegible] of the experiment, after an initial 4-h sampling period from 08.00 h, [illegible]animals were injected with 10 mg cortisol (see Section 3.4) at half-

hourly intervals for 4 h. Cortisol was injected in a volume of 2 ml heparinized saline, down the contralateral venous cannula from the one used for blood sampling. Blood samples were taken at 10-min intervals for 12 h for the estimation of LH and cortisol levels.

The mean plasma LH concentration during the 4 h of half-hourly cortisol injections was compared using Neuman-Keuls test with the mean plasma LH concentration during the pre-injection 4-h control period.

6.2.3. Cortisol infusions

As the cortisol concentrations we obtained after intravenous injection of cortisol were unsatisfactory with respect to both level and profile (see Section 6.3.2 and Figure 6.6), we decided to infuse cortisol, in an attempt to reach the plasma concentrations of cortisol that we had observed after central LHRH administration (see Section 6.3.1). Two regimes of cortisol administration were chosen for infusion, as described below. For the low dose experiment, five ewes that had been ovariectomized 6–12 months previously were used during March 1989. Eight ewes, ovariectomized at least one month earlier, were used for the high dose experiment during November 1988.

On the day before experimentation, bilateral jugular venous cannulae, kept patent with heparinized saline, were inserted into each group of long-term ovariectomized Scottish Blackface ewes. On the day of infusion, after an initial 2-h sampling period starting at 08.00 h, the animals were infused

ɔne jugular vein for 10 h at a rate of 2 ml/h with either saline or one wo doses of hydrocortisone sodium hemisuccinate dissolved in ɪrinized saline. The low-dose group of animals (n = 5) received an i.v. :tion of 1 mg cortisol, immediately followed by infusion of cortisol at 5 'h for 10 h. The other, high-dose group (n = 8), received a 6-mg i.v. :tion of cortisol, immediately followed by a 10-h cortisol infusion at 25 'h. The animals in both groups also received an infusion of saline and served as their own controls. After 9.5 h infusion, the animals were n an intravenous injection of 500 ng LHRH in saline. Plasma cortisol measured in 20-min blood samples and plasma LH in 10-min samples n from the contralateral vein.

'or the infusion experiments, precision syringe drivers (Infors HT idor type 5003, Infors AG, Bottmingen, Switzerland) were used and ɛ adjusted to deliver 2.0 ml/h, when used with 20-ml syringes. Each d control up to three such syringes, and two or three drivers were

used in each experiment. The syringes were connected to the jugular (infusion) cannulae by 210 cm of polyethylene manometer connecting tubing (as described in Section 3.3.1), taking care to ensure there were no kinks or air bubbles. Before the infusion started, the lines were filled with infusate, so that infusion of the test solution started immediately when the syringe driver was switched on. Injections were made into the infusion cannula, by momentarily disconnecting the infusion line from the cannula, immediately prior to the start of the infusion.

The mean plasma LH concentration during the first 9 h of the cortisol infusion was compared using Neuman-Keuls test with the mean LH level during the first 9 h of infusion of saline in the same group of animals. The mean area under the LH curve after intravenous LHRH injection during infusion of each dose of cortisol was also compared with the effect of LHRH injection during the control infusion of saline in the same animals.

6.3. Results

6.3.1. Intracerebroventricular experiments

Throughout the 12-h sampling period, all animals receiving saline showed a pulsatile pattern of LH secretion typical of an ovariectomized ewe and cortisol levels did not depart significantly from a baseline of about 10–12 ng/ml.

Figures 6.1 and 6.2 show the plasma LH and cortisol concentrations for each i.c.v. injection for each treatment in two ovariectomized ewes during the 12-h sampling period. Injection of saline into the third ventricle had no effect on either LH secretion or on plasma cortisol (Figures 6.1a and 6.2a). However, injection of 21 pmol LHRH resulted in a rapid rise in plasma cortisol to approximately 4–5 times pre-injection levels, and after a delay, inhibited the secretion of LH (Figures 6.1b and 6.2b). When the same dose of LHRH was injected into animals that had received a central injection of LHRH antagonist (69 pmol) 15 min earlier, the ability of third ventricular LHRH to increase plasma cortisol and to inhibit LH secretion was effectively abolished (Figures 6.1d and 6.2d). This dose of antagonist, when injected i.c.v. alone, had no effect on either plasma cortisol or LH (Figures 6.1c and 6.2c). The effects of each treatment on mean plasma levels of cortisol are shown in Figure 6.3 for all five animals. Figure 6.3a

Figure 6.1. Concentrations of LH in 10-min plasma samples and of cortisol in 30-min samples obtained over a 12-h sampling period in an individual ewe (No. 6E94), showing the marked rise in plasma cortisol after i.c.v. LHRH and the delayed inhibition of LH secretion. (a) effect of i.c.v. saline (50 μl). (b) effect of i.c.v. LHRH (21 pmol). Pretreatment with LHRH antagonist (injected i.c.v. 15 min before LHRH, twice) prevented both the rise in cortisol and the inhibition of LH. (c) effect of i.c.v. LHRH antagonist (69 pmol) alone. (d) effect of i.c.v. LHRH (21 pmol) in the presence of i.c.v. LHRH antagonist (69 pmol). Injections were made into the third ventricle at the times indicated by the arrows.

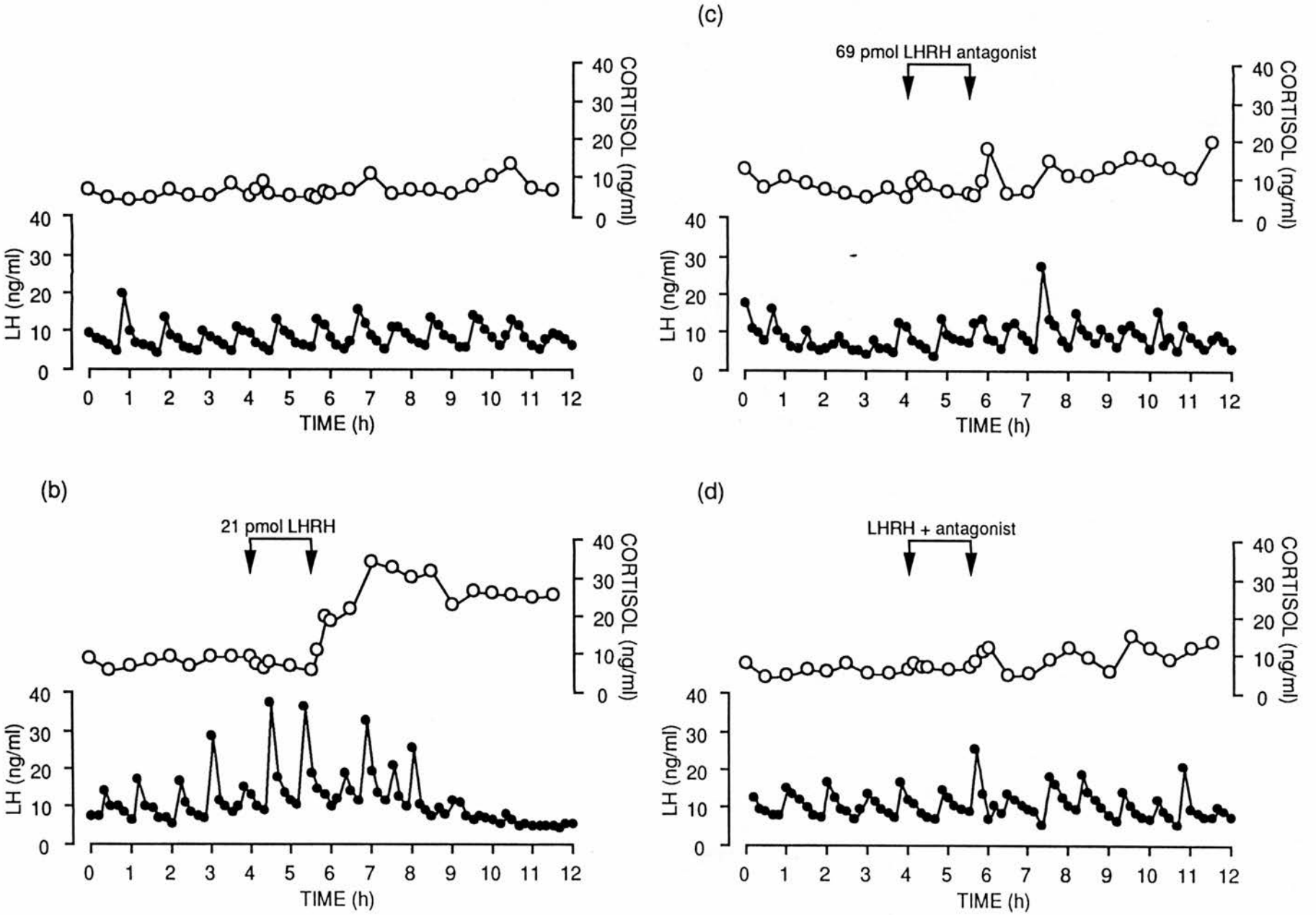

(a)
(b)
(c)
(d)
21 pmol LHRH
69 pmol LHRH antagonist
LHRH + antagonist
LH (ng/ml)
CORTISOL (ng/ml)
TIME (h)
0
10
20
30
40
0 1 2 3 4 5 6 7 8 9 10 11 12

Figure 6.2. Concentrations of LH in 10-min plasma samples and of cortisol in 30-min samples obtained over a 12-h sampling period in another ewe (No. 115). (a) effect of i.c.v. saline (50 μl). (b) effect of i.c.v. LHRH (21 pmol). (c) effect of i.c.v. LHRH antagonist (69 pmol) alone. (d) effect of i.c.v. LHRH (21 pmol) in the presence of i.c.v. LHRH antagonist (69 pmol). See legend of Figure 6.1 for further details, but note that scales of axes differ. (Three of this animal's LH profiles have already been shown in Figure 4.6, but are presented again for ease of comparison).

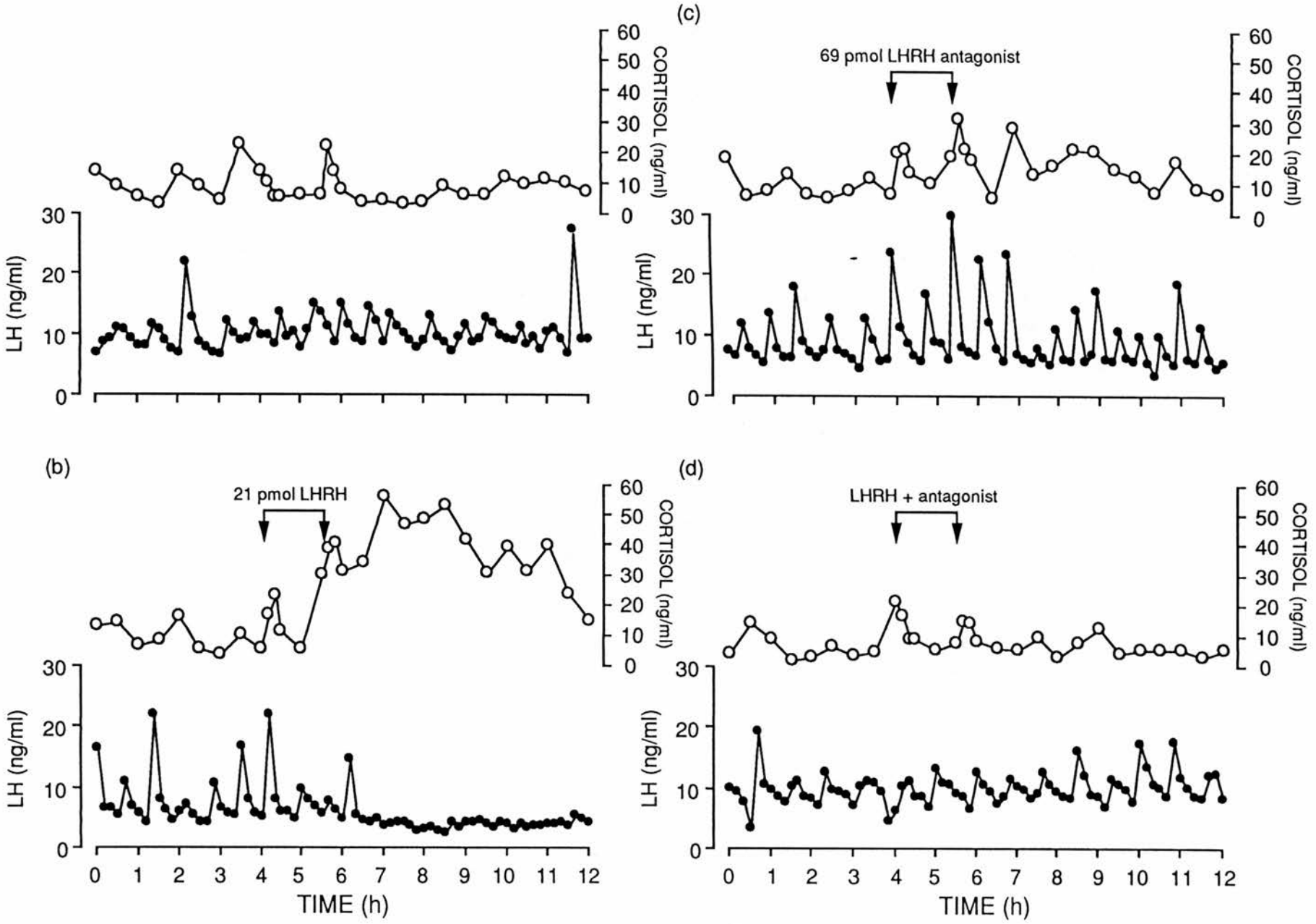
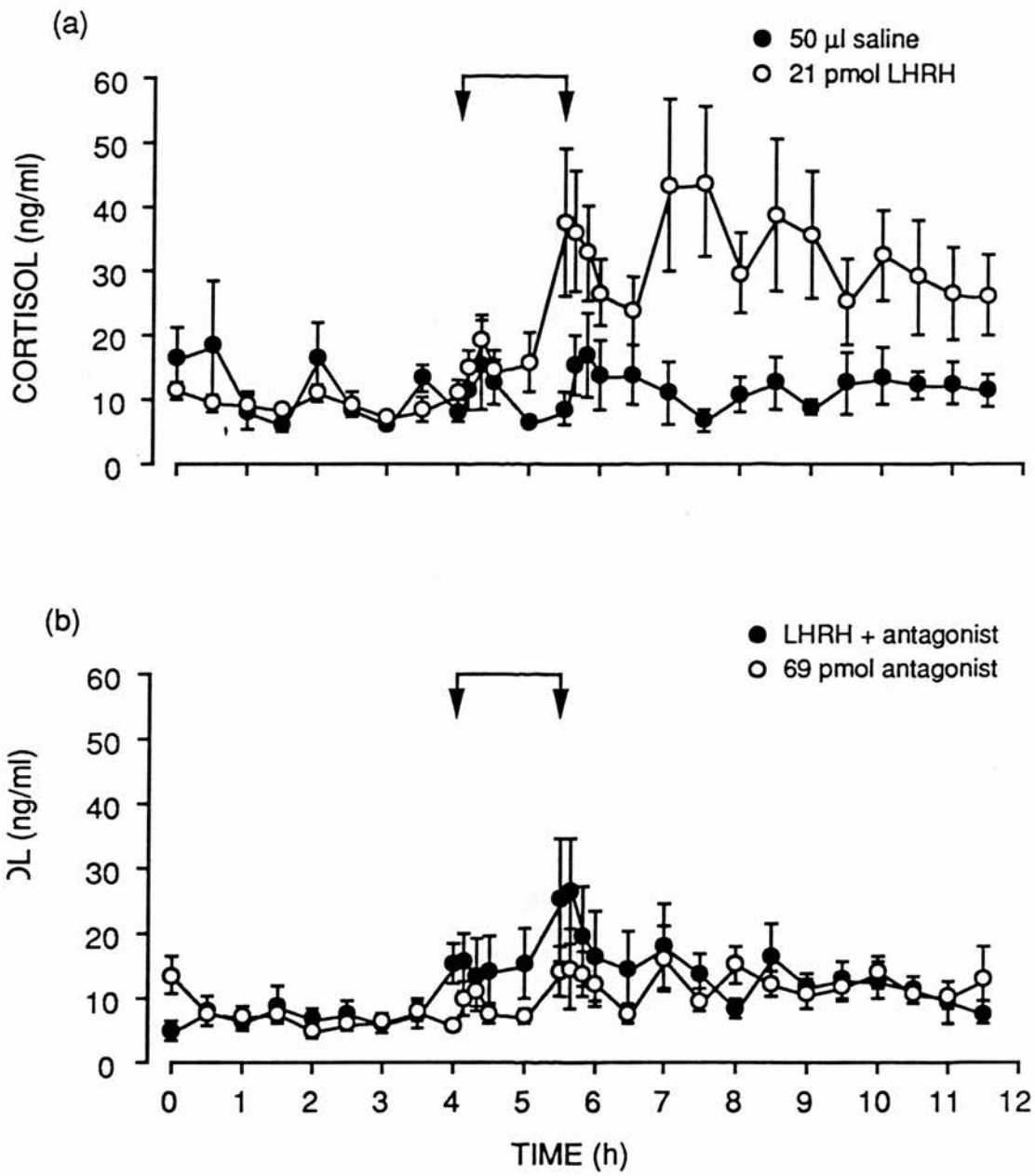

(c)
69 pmol LHRH antagonist
(b)
21 pmol LHRH
(d)
LHRH + antagonist
CORTISOL (ng/ml)
LH (ng/ml)
TIME (h)

Figure 6.3. Effects of four treatments on mean half-hourly plasma levels of cortisol, showing the stimulatory effect of central LHRH on plasma cortisol and the ability of an LHRH antagonist to block this. (a) effects of i.c.v. saline (50 μl) and of i.c.v. LHRH (21 pmol). (b) effects of LHRH antagonist (69 pmol) and of LHRH (21 pmol) in the presence of antagonist (69 pmol). Times of injection are indicated by arrows. Values are expressed as the mean ± s.e.m. (n = 5).


(a)
50 µl saline
21 pmol LHRH
CORTISOL (ng/ml)
60
50
40
30
20
10
0
(b)
LHRH + antagonist
69 pmol antagonist
ɔL (ng/ml)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
TIME (h)

shows the effect of central injection of 21 pmol LHRH on mean plasma cortisol concentrations, compared with the control response to saline. In Figure 6.3b, the ability of the antagonist to block the stimulatory effect of LHRH on cortisol secretion is shown, as is the lack of effect of LHRH antagonist alone. For clarity, the effect of the lower (2.1 pmol) dose of LHRH on cortisol and LH secretion has been omitted from these first three figures.

The effects of each of the treatments on plasma cortisol secretion, expressed as the area under the cortisol curve, are shown in Figure 6.4 for all five animals. Analysis of variance, followed by Neuman-Keuls test, showed there to be no significant difference between the areas under the cortisol curves in the 3.5-h pre-injection sampling period (Figure 6.4a). In addition, there was no significant difference between the rises in plasma cortisol seen after 21 pmol LHRH in the two 3.5-h post-injection sampling periods. These data are therefore presented together (Figure 6.4b). Intracerebroventricular injection of 21 pmol LHRH significantly increased the area under the cortisol curve in the 7-h post-injection period compared with saline ($p < 0.01$) and with LHRH antagonist ($p < 0.01$). The LHRH antagonist was able to block the ability of central LHRH to increase cortisol: when 21 pmol LHRH was injected i.c.v. 15 min after 69 pmol LHRH antagonist, also administered i.c.v., the area under the cortisol curve was similar to that after saline and significantly different from that after 21 pmol LHRH ($p < 0.05$). The increase in cortisol secretion by central LHRH was dose-related in that a 10-fold lower dose (2.1 pmol) only ·ased the area under the cortisol curve significantly in the first 3.5-h ·injection sampling period whereas the increase in the area under the sol curve did not reach significance in the second (data not shown). hapter 4, it was shown that LH secretion was not reduced until the . 3.5-h time period (see Figure 4.4). If the increase in cortisol secretion mpared with the decrease in LH secretion during this time, there is a degree of correlation. This is shown in Figure 6.5 where the change ea under the LH curve is plotted against the change in area under the sol curve at this time for both doses of LHRH. The correlation icient, r, was –0.902, indicating that the higher the increase in plasma sol, the greater the reduction in LH.

. Cortisol injections

venous injection of 10 mg of cortisol at half-hourly intervals resulted

Figure 6.4. Grouped data showing the effects of each treatment on the mean area under the cortisol curve (± s.e.m.). Doses of LHRH and LHRH antagonist as shown. Where animals were treated with both LHRH and LHRH antagonist, the dose of LHRH used was 21 pmol. (a) area under cortisol curve in the 3.5-h time period prior to the first i.c.v. injection for each treatment. (b) area under the cortisol curve in the 7 h after the first injection (data pooled from the two post-injection 3.5-h time periods). Note changed scale of y-axis. Levels of significance are shown (n = 5 for each treatment).

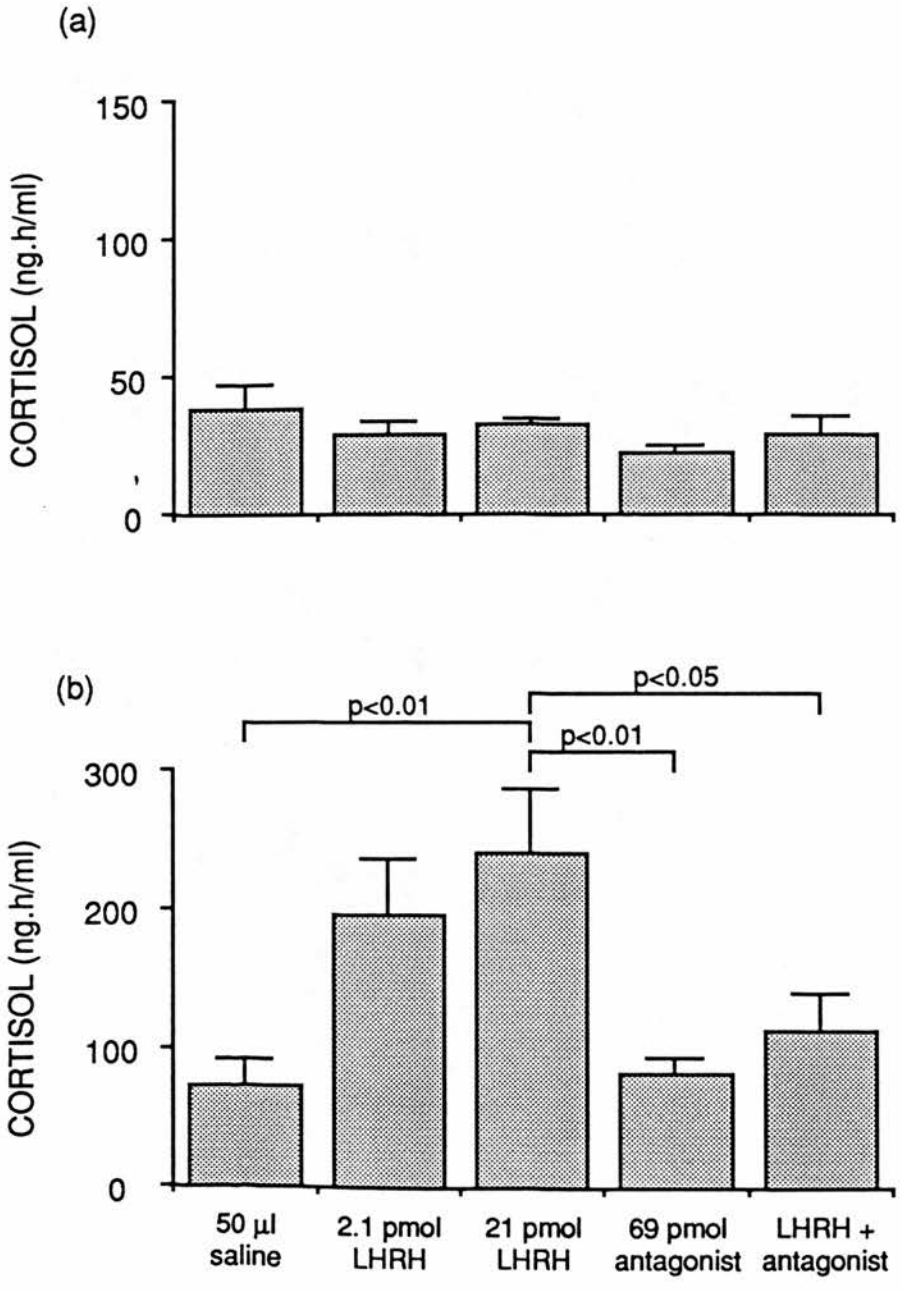
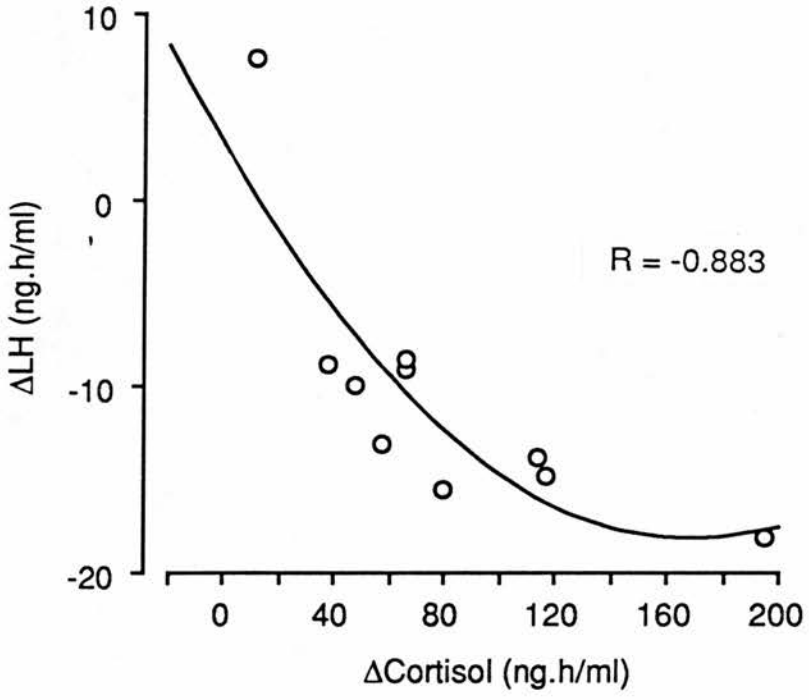

(a)
150
100
50
0
CORTISOL (ng.h/ml)
(b)
p<0.01
p<0.05
p<0.01
300
200
100
0
CORTISOL (ng.h/ml)
50 µl saline
2.1 pmol LHRH
21 pmol LHRH
69 pmol antagonist
LHRH + antagonist

Figure 6.5. Relationship between change in area under cortisol curve (x-axis) and change in area under LH curve (y-axis) for each individual animal comparing the third 3.5-h sampling period with the 3.5-h pre-injection control period. O = i.c.v. LHRH-treated animals (both doses shown) (n = 5 for each treatment).


10
0
-10
-20
ΔLH (ng.h/ml)
R = -0.883
0
40
80
120
160
200
ΔCortisol (ng.h/ml)

in sharp peaks of cortisol in plasma, reaching up to ~300 ng/ml and declining rapidly to ~100 ng/ml within 0.5 h. Figure 6.6b shows the mean plasma cortisol concentrations in samples taken every 10 min for three ewes as a result of half-hourly cortisol injection. When cortisol was measured only in 20-min samples, half of the cortisol peaks were missed (Figure 6.6a). This highlights the fact that there is no cortisol binding globulin in the sheep and shows the importance of careful interpretation of results. The plasma cortisol profile created by the intravenous injection of cortisol was thus not of the same pattern or level as we had measured after i.c.v. injection of LHRH. There was no effect on plasma concentrations of LH (Figure 6.6c).

6.3.3. Cortisol infusions

Intravenous injection of 1 mg cortisol followed by infusion of cortisol at 5 mg/h for 10 h achieved plasma cortisol levels of 40–50 ng/ml (Figure 6.7a), i.e., comparable to the raised levels of cortisol that we observed after central LHRH administration (see Figures 6.1b and 6.2b). This dose reduced LH levels significantly ($p < 0.05$) when compared with the effect of the control infusion of saline (Figure 6.8). Intravenous injection of 6 mg cortisol followed by infusion at 25 mg/h for 10 h had a greater effect on LH levels. This dose achieved a plasma cortisol level of 300–400 ng/ml (Figure 6.7b), i.e., ~8-fold higher than we saw after central LHRH administration and 20- to 30-fold higher than physiological levels. Mean
levels were reduced significantly compared with both saline control (p
)1) and the lower dose of cortisol ($p < 0.01$) (Figure 6.8). This reduction
H secretion was more gradual than after central LHRH administration.
ı doses significantly reduced the LH response of the pituitary gland to
ıtravenous bolus dose of 500 ng LHRH given 0.5 h before the end of
nfusion ($p < 0.05$) (Figure 6.9).

Discussion

:tion of LHRH into the third cerebral ventricle of the ovariectomized
caused a significant and rapid rise in plasma cortisol concentration
:h was prevented by prior treatment with an LHRH antagonist. The
ulatory effect of i.c.v. LHRH was dose-related in that the lower dose
:ased cortisol secretion initially post-injection whereas the effect of the

Figure 6.6. Effects of intravenous injection of 10 mg cortisol at half-hourly intervals for 4 h on plasma cortisol and LH levels. Injections commenced at t = 4 h as described in the text. (a) measurement of cortisol concentrations in 20-min samples misses half the peaks. Data shown as mean ± s.e.m. (n = 6). (b) cortisol levels re-assayed in 10-min samples for three of the ewes. The half-life of cortisol in sheep plasma is very brief, and injections cannot sustain a plateau. (c) effect of cortisol injections on mean area under LH curve (± s.e.m.) comparing 4 h before with 4 h during injections (n = 6). Effect of control injections of saline also shown. Intravenous injection of cortisol did not affect LH secretion.

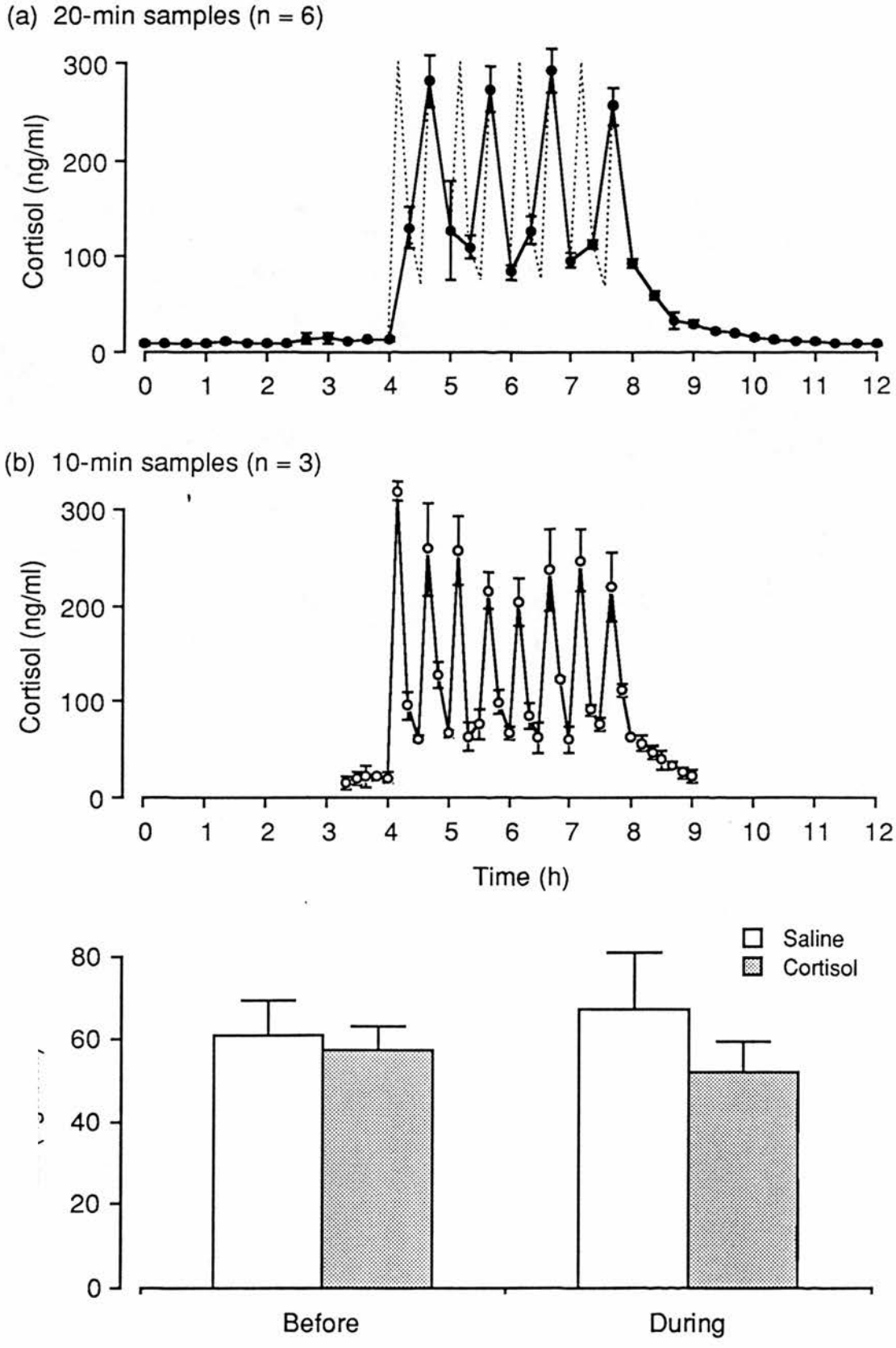

(a) 20-min samples (n = 6)
Cortisol (ng/ml)
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12
(b) 10-min samples (n = 3)
Cortisol (ng/ml)
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (h)
Saline
Cortisol
80
60
40
20
0
Before
During

Figure 6.7. Effects of 10 h intravenous infusion of hydrocortisone sodium hemisuccinate or saline on mean plasma cortisol concentrations. The period of infusion is represented by the stippled bar and starts at t = 2 h. (a) injection of 1 mg cortisol i.v. at arrow (t = 2 h), followed by 10 h infusion at 5 mg/h (n = 5). (b) injection of 6 mg cortisol i.v. at arrow (t = 2 h), followed by 10 h infusion at 25 mg/h (n = 8) (note change of scale). Values are expressed as the mean ± s.e.m. The plasma cortisol levels during control infusion of saline in each group are shown for comparison (note that normal secretion of cortisol is pulsatile).

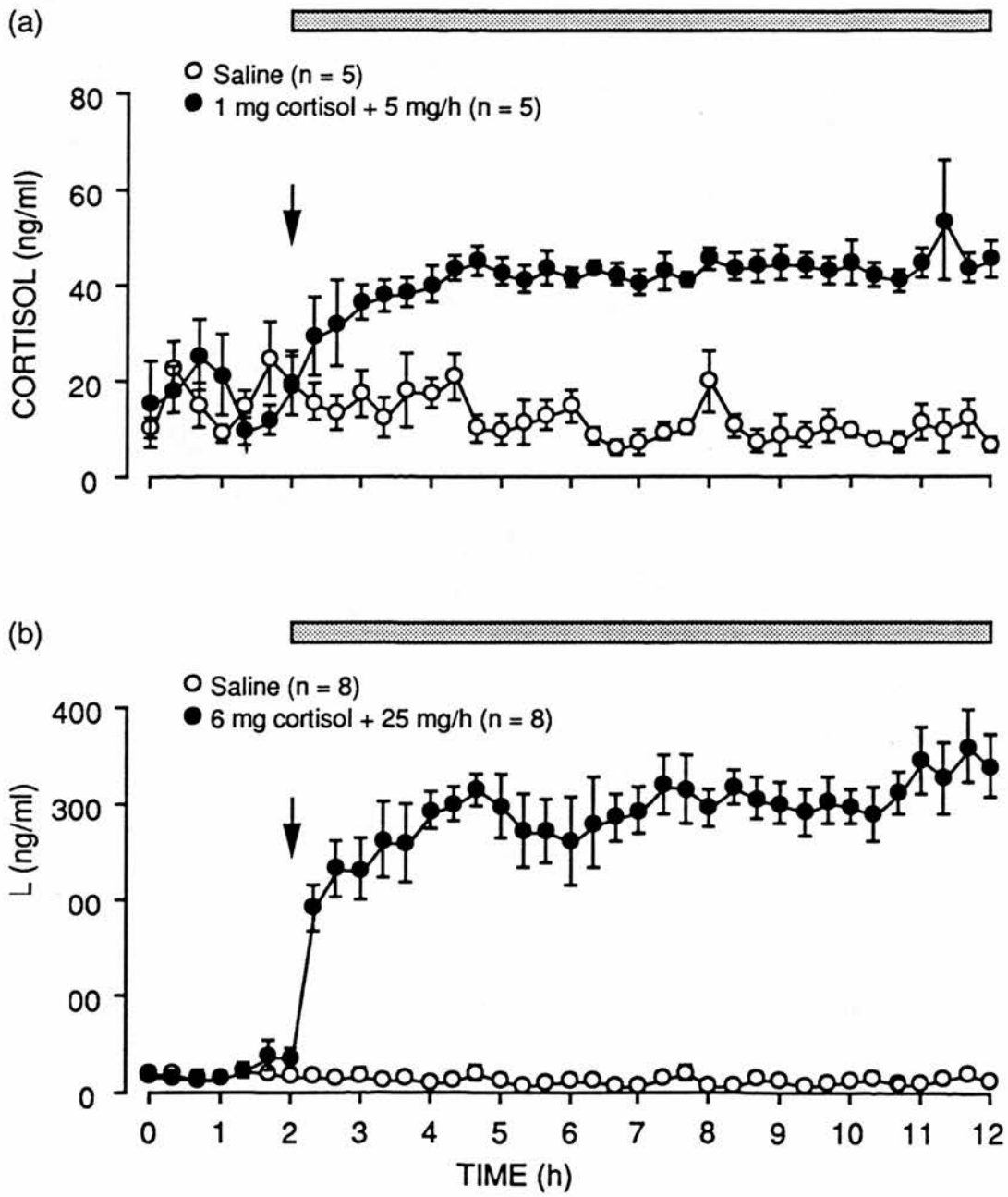
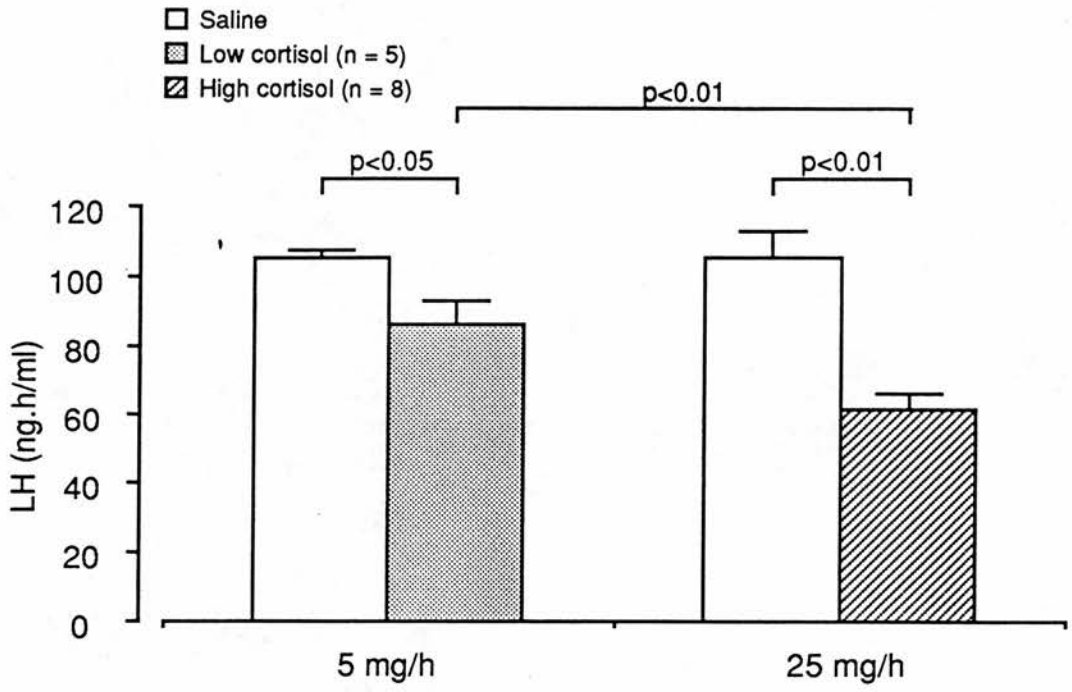
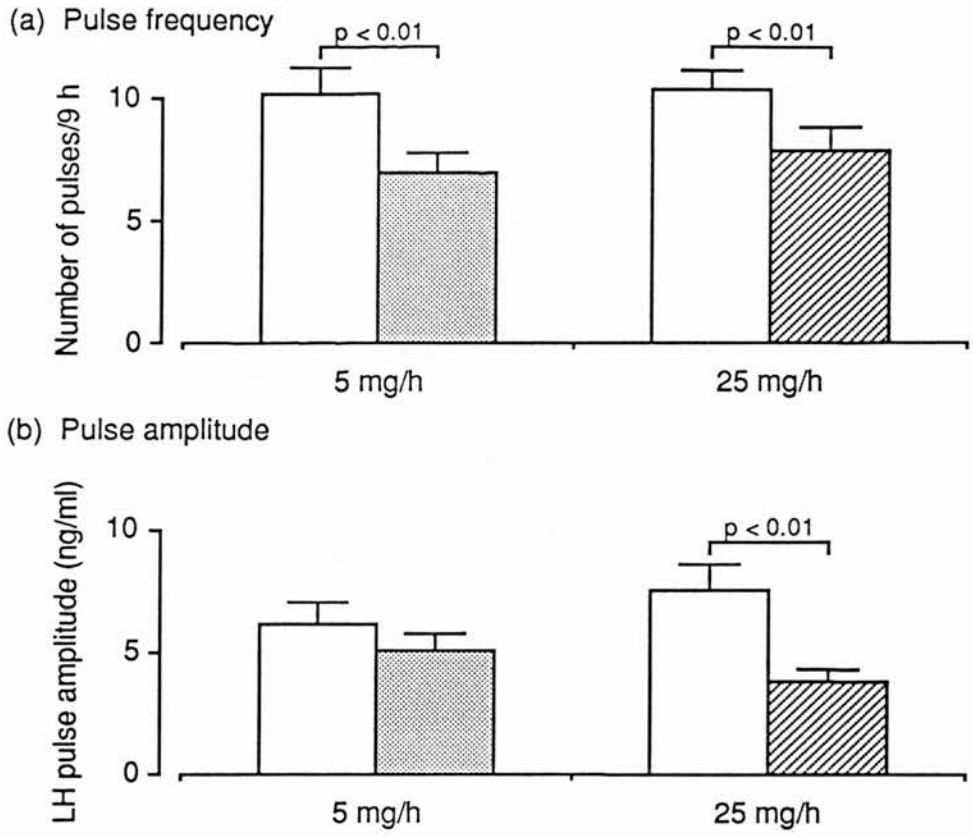

(a)
Saline (n = 5)
1 mg cortisol + 5 mg/h (n = 5)
CORTISOL (ng/ml)
80
60
40
20
0
(b)
Saline (n = 8)
6 mg cortisol + 25 mg/h (n = 8)
L (ng/ml)
400
300
00
00
0
0 1 2 3 4 5 6 7 8 9 10 11 12
TIME (h)

Figure 6.8. Effect of cortisol or saline infusion on mean area under LH curve (± s.e.m.) for first 9 h of infusion. Left; injection of 1 mg cortisol i.v. followed by infusion at 5 mg/h (n = 5). Right; injection of 6 mg cortisol i.v. followed by infusion at 25 mg/h (n = 8). Effects of control infusion of saline in each group are also included. Levels of significance are shown.

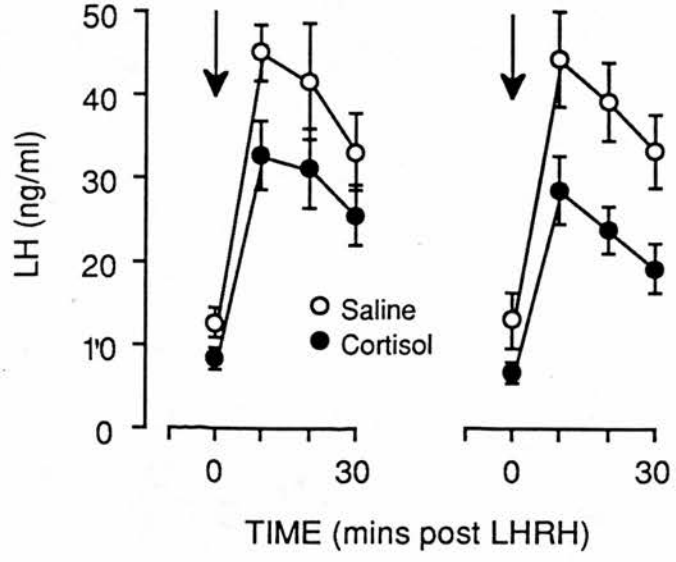

Saline
Low cortisol (n = 5)
High cortisol (n = 8)
p<0.01
p<0.05
p<0.01
120
100
80
60
40
20
0
LH (ng.h/ml)
5 mg/h
25 mg/h



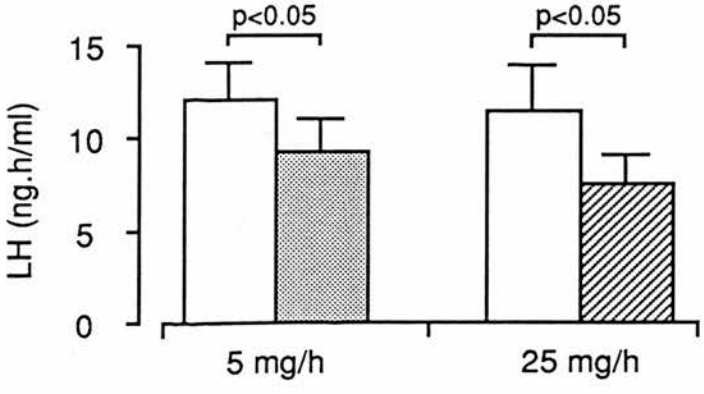
Addendum: Figure 6.8.1. Effect of cortisol or saline infusion on (a) LH pulse frequency (± s.e.m.) and (b) LH pulse amplitude (± s.e.m.) for first 9 h after infusion. See Figure 6.8 for further details.

Figure 6.9. Effect of infusion of cortisol on ability of LHRH to release LH from the pituitary gland. 500 ng LHRH given i.v. 0.5 h before end of infusion, i.e., at t = 11.5 h. (a) effect of i.v. injection of 500 ng LHRH (at arrow) on LH release during (left) infusion of cortisol at 5 mg/h (n = 5) and (right) infusion of cortisol at 25 mg/h (n = 8). Plasma concentrations of LH expressed as mean ± s.e.m. (b) both doses significantly reduced the mean area under the LH curve, after correction for baseline LH secretion ($p < 0.05$).

(a)


50
40
30
20
10
0
LH (ng/ml)
Saline
Cortisol
0
30
0
30
TIME (mins post LHRH)

(b)


p<0.05
p<0.05
15
10
5
0
LH (ng.h/ml)
5 mg/h
25 mg/h

higher dose was more sustained. The rise in plasma cortisol was followed by a reduction in plasma LH secretion as described in Chapter 4. The degree of suppression of LH secretion correlated highly with the increase in cortisol. Whilst intravenous injection was without effect, intravenous infusion of cortisol to reach the same levels as observed after central LHRH administration also significantly reduced LH secretion, but not to the extent achieved after central LHRH. Furthermore, during the infusion of cortisol, the response of the pituitary gland to an intravenous bolus injection of LHRH was reduced, suggesting that the inhibitory effect of cortisol infusion on LH secretion is due, at least in part, to a reduced pituitary responsiveness to LHRH.

Pituitary responsiveness to i.v. LHRH was not, however, compromised by the central LHRH treatment (Chapter 4). It could be argued that as we observed an apparent reduction in pituitary LHRH-responsiveness with infusion of cortisol, even at the lower dose, then this suggests that cortisol is not a causative factor in the LHRH-induced inhibition of LH secretion. Indeed, this may be the case, but it should be pointed out that although the aim of the infusion experiments was to try to re-create the plasma levels of cortisol observed after central LHRH administration, the actual pattern achieved was different in several ways. The infusion experiments created a much more rapid rise in plasma cortisol concentration which then remained at a uniformly high level throughout the following 10 h. The rise in cortisol after central LHRH was not sustained at such a constant [illegible] and was beginning to tail off towards the end of the sampling period, h later. Thus the test of pituitary LHRH responsiveness during the ion was performed after almost 9.5 h of sustained increase in cortisol, eas after the i.c.v. experiments, pituitary responsiveness was tested after the first i.c.v. injection, i.e. perhaps 6–7 h after the initial vement of the high concentrations. These observations, of course, do preclude a contributory role for cortisol as it is quite conceivable that sol may have profound and rapid effects at the level of the thalamus and either delayed effects on the pituitary gland, or effects are dependent on a more sustained rise. In this regard, it is esting to note that when we gave half-hourly injections of cortisol to iectomized ewes in the preliminary experiment, these had no effect lasma LH concentrations.

lthough the stimulation of cortisol release by third ventricular LHRH cts the activation of the hypothalamo-pituitary-adrenal axis, it does

not represent a non-specific 'stress' response as neither saline nor the antagonist when injected alone affected cortisol secretion. In addition, the failure of the LHRH antagonist to affect the secretion of cortisol, at the dose tested, suggests that the hypothalamo-pituitary-adrenal axis is not under tonic control by LHRH.

The aim of the experiments described in this chapter was to try to investigate the delayed and sustained nature of the LHRH-induced inhibition of LH secretion. Although it is possible that this delay could be due, for example, to the time taken for the LHRH to diffuse from the third ventricle to its site of action this is unlikely as presumably the LHRH would be subject to degradation by proteases in the CSF and extracellular spaces of the brain. The sustained nature of the inhibition could be due to LHRH acting on a central receptor with a greater affinity for LHRH than has the classical' pituitary receptor. Although we have no evidence to support or refute this possibility, it seems unlikely as the inhibitory effect of LHRH on LH secretion was readily blocked by the prior administration of an LHRH antagonist. One more interesting possibility which would account for these findings is that the hypothalamo-pituitary-adrenal axis is acting to affect the reproductive axis. The high degree of correlation between the increase in plasma cortisol and the reduction in LH secretion suggests that the two responses are related. As the reduction in LH secretion took longer to become evident than the increased cortisol concentration, it is tempting to postulate a causative link; i.e., that the stimulation of the hypothalamic-adrenal axis by central LHRH results in a

ction in the activity of the LHRH/LH system.

lthough achieving similar plasma levels does not necessarily reflect ing relevant concentrations at the active site(s) of a drug, intravenous ion of cortisol to reach the plasma concentrations seen after third icular LHRH administration reduced plasma LH levels significantly. ever, as we did not achieve a reduction to levels of LH obtained after ventricular LHRH administration and as the reduction in LH tion that we did observe during the infusion was not so abrupt, it ars that the action of cortisol alone may not account for the whole omenon. Although, as we were unable to measure ACTH, we ot discount the intriguing possibility that we have activated a direct al input to the adrenal cortex, the LHRH-induced stimulation of sol secretion presumably reflects an increase in activity of the thalamo-pituitary-adrenal axis. Therefore, in addition to the

increased levels of cortisol reported in this chapter, endogenous CRH, AVP and ACTH levels are all likely to be raised during this time and may contribute to the large decrease in LH. It may be, for example, that centrally-administered LHRH stimulates CRH release and that CRH itself acts to suppress the hypothalamo-pituitary-gonadal axis. There is much evidence to support this hypothesis for the rat. For instance, i.c.v. CRH inhibits plasma LH secretion in the rat (Rivier & Vale, 1984), presumably by inhibiting LHRH release from the mediobasal hypothalamus and the median eminence. The latter mechanism, which is CRH-receptor-mediated, has been demonstrated for the male rat *in vitro* (Gambacciani, Yen & Rasmussen, 1986). In addition, LHRH release into the hypothalamo-pituitary portal system decreases after i.c.v. administration of CRH in female rats (Petraglia, Sutton, Vale & Plotsky, 1987). There is also recent immunocytochemical evidence in the rat for direct synaptic contacts between CRH- and LHRH-containing neurones in the medial preoptic area (MacLusky, Naftolin & Leranth, 1988). These lines of evidence suggest that, at least in the ovariectomized rat, CRH can act centrally to inhibit gonadotrophin release. However, i.c.v. injection of CRH in the ovariectomized ewe results in either no effect (Horton, Francis & Clarke, 1988) or a stimulation of LH secretion (Naylor, Porter & Lincoln, 1990) as described in the following chapter. Therefore, an increase in CRH is unlikely to account for these results in the sheep. This could, however, be investigated further by pretreatment with $\alpha CRH_{(9-41)}$ antagonist or by ve immunization against CRH peripherally or centrally.

ne other attractive possibility is that the centrally injected LHRH is g as a neurotransmitter with regard to the hypothalamic-ovarian and ypothalamic-adrenal axes. In other words, this LHRH may be acting imic the influence of LHRH neurones from higher centres both to ılate the hypothalamo-pituitary-adrenal axis and to inhibit the ɔductive axis. It may be therefore that the increase in cortisol is just a ıidental phenomenon and not causally related to the suppression of ecretion. However, there is much evidence that increased activity of ypothalamo-pituitary-adrenal axis in general, e.g. under conditions of ical and environmental stress, and that increased plasma levels of ɔcorticoids in particular will adversely affect reproduction. Firstly, is a clear correlation between plasma cortisol levels and suppression ɔnadotrophin secretion in patients with Cushing's disease (White, erson, Mashiter & Joplin, 1981). Secondly, physical stress, such as

unaccustomed strenuous exercise can result in menstrual disorders (Bullen, Skrinar, Beitins *et al.* 1985): female runners, who often have amenorrhoea, have increased plasma cortisol levels (Villanueva, Schlosser, Hopper *et al.* 1986), as do women with anorexia nervosa (Gold, Gwirtsman, Avgerinos *et al.* 1986) and patients with functional hypothalamic amenorrhoea (Suh, Liu, Berga *et al.* 1988). Thirdly, administration of glucocorticoids in large doses, as in the treatment of asthma or of chronic inflammatory disorders, may interrupt normal menstrual cyclicity and render patients subfertile (Cunningham, Caperton, Goldzieher, 1975; Sakakura, Takebe, Nakagawa, 1975).

In the sheep, cortisol and/or ACTH has been reported to influence LH secretion in a number of situations. The LHRH- and oestradiol benzoate-induced release of LH is suppressed in the ewe by intravenous ACTH (Dobson, Essawy & Alam, 1988). This is in contrast to the effects of i.v. ACTH infusion in the ovariectomized monkey (Xiao & Ferin, 1988): in this experimental model the secretion of LH and FSH is unaffected. Although intravenous infusion of cortisol has been shown to have no effect on the oestradiol-induced LH surge in ewes (Moberg, Watson, Stoebel & Cook, 1981) the animals investigated were intact anoestrous ewes unlike the ovariectomized animals used in the current study. These workers have also shown that in the ram, ACTH (but not short-term cortisol) suppresses the LH response to i.v. LHRH (Fuquay & Moberg, 1983; Matteri, Watson & Moberg, 1984). It would appear, however, that the

ts of ACTH may, at least in the rat, be mediated via adrenal factors and ria ACTH itself, since adrenalectomy abolishes the ability of ACTH to ce plasma LH levels and i.c.v. ACTH has no effect on LH secretion in, Evans, Edoimioya *et al.* 1985). There is evidence, also, that in ans the ability of ACTH to lower basal LH levels is mediated through cute rise in plasma cortisol (Vierhapper, Waldhäusl & Nowotny,). Both ACTH and cortisol therefore seem able to act on the othalamus and/or the anterior pituitary to interfere with dotrophin release. Whilst we cannot make any definitive statements, a possibility that in the present study we have observed a biphasic n of cortisol; i.e., a rapid hypothalamic effect followed by an effect on ituitary gland that is observed after a sustained rise in plasma cortisol. conclusion, central administration of LHRH caused a significant and dose-related rise in plasma cortisol concentration in the ectomized ewe. This was associated with a delayed but sustained

reduction in LH secretion, the degree of reduction in LH correlating highly with the increase in cortisol. Intravenous infusion of cortisol, to reach plasma levels which were seen after central LHRH, significantly reduced LH secretion due in part to a reduction in LHRH-responsiveness at the pituitary gland. However, the pulsatile secretion of LH was not reduced to that observed following central LHRH administration. These experiments suggest that LHRH acts as a central neurotransmitter to activate the hypothalamo-pituitary-adrenal axis and that cortisol, alone or in conjunction with another component(s) of the hypothalamo-pituitary-adrenal axis, may play a role in the LHRH-induced inhibition of LHRH/LH secretion (LHRH autoregulation) in the sheep.

7. Effects of central administration of corticotrophin-releasing hormone on the secretion of gonadotrophins, prolactin and cortisol

7.1. Introduction

The experiments reported in this chapter develop further the theme of the interaction between the hypothalamic-adrenal and hypothalamic-ovarian axes. In the previous chapter, it was demonstrated that LHRH acts as a neurotransmitter to stimulate the hypothalamo-pituitary-adrenal axis. In addition, while we could not conclusively show it to be responsible for the LHRH-induced suppression of LH, there is a profound effect of the hypothalamo-pituitary-adrenal axis on the reproductive axis.

There is much evidence, especially in the rat, but also in other species including the human, that stress of various types disrupts the normal secretion of reproductive hormones and therefore exerts a deleterious effect on reproductive function (Gray, Smith, Damassa *et al.* 1978; Du Ruisseau, Taché, Brazeau & Collu, 1978; Taché, Du Ruisseau, Ducharme & Collu, 1978; Rasmussen & Malven, 1983; Bullen, Skrinar, Beitins *et al.*
Gold, Gwirtsman, Avgerinos *et al.* 1986; Rivier, Rivier & Vale, 1986).
stress increases the plasma concentration of adrenocorticotrophic
one and of glucocorticoids, it is often suggested that activation of the
thalamo-pituitary-adrenal axis is responsible for stress-induced
ility. As discussed in the previous chapter, there is well-documented
nce for a role of each of the components of this axis in the
ession of LHRH and LH secretion. For example, administration of
I and cortisol delays puberty and inhibits ovulation in rats (Smith,
on, Weick *et al.* 1971; Baldwin & Sawyer, 1974; MacFarland & Mann,
Baldwin, 1979), interrupts normal menstrual cyclicity in humans
ıingham, Caperton, Goldzieher, 1975; Sakakura, Takebe, Nakagawa,
and interferes with gonadotrophin release through an action on the
halamus and/or the anterior pituitary gland (Moberg 1976; Moberg,
ɔn, Stoebel & Cook, 1981; Fuquay & Moberg, 1983; Matteri, Watson &

Moberg, 1984; Dubey & Plant, 1985; Dobson, Essawy & Alam, 1988).

In addition to the actions of ACTH and cortisol, corticotrophin-releasing hormone can act at the level of the central nervous system to inhibit LH release in ovariectomized, adrenalectomized rats (Rivier & Vale, 1984) and ovariectomized monkeys (Olster & Ferin, 1987; Gindoff & Ferin, 1987). The inhibitory effect of central administration of CRH on gonadotrophin release is due to inhibition of LHRH release into the hypophysial portal circulation (Petraglia, Sutton, Vale & Plotsky, 1987), and is mediated by endogenous opioid peptides (Gindoff & Ferin, 1987; Almeida, Nikolarakis & Herz, 1988; Nikolarakis, Almeida & Herz, 1988; Nikolarakis, Almeida, Sirinathsinghji & Herz, 1988).

Since the central injection of a CRH antagonist such as $\alpha CRH_{(9\text{-}41)}$ (Rivier, Rivier & Vale, 1984) can reverse the stress-induced suppression of LH secretion in the rat (Rivier, Rivier & Vale, 1986), it has been proposed that endogenous central CRH may mediate the deleterious effects of stress on LHRH/LH release by an effect distinct from its activation of the pituitary-adrenal axis. The hypothesis that endogenous CRH may participate as a neurotransmitter in the brain and be responsible for mediating responses to stress is strengthened further by the following observations. Firstly, intracerebroventricular administration of the hormone elicits prolonged increases in plasma concentrations of noradrenaline and adrenaline (Brown, Fisher, Rivier *et al.* 1982; Brown, Fisher, Webb *et al.* 1985; Brown, Gray & Fisher, 1986) similar to those
-'- -ved during stress. Secondly, there is evidence that exogenous and
genous CRH may be involved in enhancing stress-induced
viours in the rat (Britton, Koob, Rivier & Vale, 1982; Krahn, Gosnell,
e & Levine, 1986; Kalin, Sherman & Takahashi, 1988).

he following studies were therefore undertaken in ovariectomized
to investigate further the hypothesis that central CRH acts as a
otransmitter mediating stress responses in the brain. The effect of
al administration of CRH on pulsatile LH and FSH secretion was
tigated. In addition, the influence of CRH on the release of two stress
ones which are under different regulatory control mechanisms,
ly prolactin and cortisol, was studied. The potential involvement of
genous opioid peptides in the actions of centrally administered CRH
nvestigated using the opioid antagonist naloxone.

7.2. Experimental design

Five Scottish Blackface ewes were housed in individual pens in the University of Edinburgh Marshall Building, near Edinburgh, under artificial lighting conditions corresponding to natural daylength from February–May 1988 and from August–October 1988. Sheep were fed concentrated pellets once a day and hay and water were available *ad libitum*.

At least three to four weeks before experimentation, the animals were ovariectomized and implanted with a stainless steel cannula directed towards the third ventricle as described previously.

In the initial group of experiments (February–May), animals received single central injections of ovine CRH (see Section 3.4) at two doses, 0.12 nmol (0.5 μg) and 1.2 nmol (5 μg), and of 50 μl saline as control. In addition, each animal received i.v. naloxone (4 x 25 mg) concomitantly with 1.2 nmol CRH and also control injections of i.v. naloxone alone. Two further treatments were given in a second group of experiments (August–October). Firstly, animals were treated with a single, higher, dose of CRH (5.2 nmol). Secondly, each animal received repeated (three) i.c.v. injections of 1.2 nmol CRH and also (three) control injections of saline.

On the day before each sampling period, a jugular venous cannula was inserted into each ewe and kept patent with heparinized saline. Blood
ıles (3 ml) were collected at 10-min intervals for 10–12 h from 08.00 h
ıe day of each experiment. After an initial 4-h control period a single
injection was made. The order of the various injections was based
cross-over design with each ewe serving as her own control and
ving each treatment. When multiple i.c.v. injections of saline or
were required, they were delivered at 3.5, 4.5, and 5.5 h into the
ıling period. Naloxone (4 x 25 mg) was injected intravenously at 4,
and 8.5 h into the sampling period.
lasma concentrations of LH were measured in samples taken every 10
FSH, prolactin and cortisol levels were determined in 20-min
ıles. For the analysis of the LH data, the sampling period was divided
three equal 3.5-h windows representing time periods before (pre),
ıg (post 1) and after (post 2) the central injections. In the case of FSH,
.ctin and cortisol, the mean areas under the curves were calculated
ıg each treatment (3 h after injection).

7.3. Results

Over the 12-h sampling period, control animals receiving saline showed the characteristic pulsatile LH secretion pattern of an ovariectomized ewe. During this period, the LH pulse frequency and mean LH concentration did not change significantly with time.

Figure 7.1 shows the plasma LH concentrations over the 12-h sampling period after injection of saline or three concentrations of CRH into the third ventricle of an individual ewe. Injection of 50 µl saline into the ventricular system did not alter the pulsatile secretion of LH (Figure 7.1a). Similarly, injection of the lowest concentration of CRH appeared to be without effect (Figure 7.1b). However, the administration of either 1.2 or 5.2 nmol CRH resulted in a stimulation of LH release (Figure 7.1c and d).

Figure 7.2 shows the grouped effects of three of these treatments on LH pulse frequency (Figure 7.2a–c) and mean LH concentration (Figure 7.2d–f) for all five animals. Central injection of 50 µl saline did not affect either LH pulse frequency (Figure 7.2a) or mean LH levels (Figure 7.2d). Following injection of 1.2 nmol CRH, however, there was a significant ($p < 0.01$) increase in the mean LH concentration (Figure 7.2f) as a consequence of a significant ($p < 0.01$) increase in LH pulse frequency (Figure 7.2c). This stimulation of LH secretion appears to be dose-related, as administration of a 10-fold lower dose (0.12 nmol) of CRH caused a

: increase in mean LH levels (Figure 7.2e), although this did not reach
tical significance. LH pulse amplitude following i.c.v. injection of
was not significantly different from the saline controls (Figure
-c), owing to large individual variation. In contrast to the effects on
ecretion, plasma levels of FSH were unaffected by central injection of
(Figure 7.3).

terestingly, in the individual animal's data shown in Figure 7.1 it
ars that the period of stimulation of LH secretion elicited by CRH is
wed by an inhibitory phase. However, such a response was not
nonly observed: in the post 2 time period, the LH pulse frequency and
LH concentrations returned to levels observed prior to CRH
nistration (Figure 7.2). Furthermore, repeated (three) injections of 1.2
CRH did not result in any suppression of LH secretion (Figure 7.4).
stimulatory effect of central administration of CRH on LH secretion
not altered by naloxone (Figure 7.2.1d–f) even though intravenous

Figure 7.1. Concentrations of LH in 10-min plasma samples obtained over a 12-h sampling period in an individual ewe (No. A124) showing the dose-related stimulation of LH secretion by centrally-injected CRH. (a) effect of i.c.v. injection with 50 µl saline. (b) effect of 0.12 nmol CRH i.c.v. (c) effect of 1.2 nmol CRH i.c.v. (d) effect of 5.2 nmol CRH i.c.v. Time of injection (t = 4 h) is indicated by the arrow. Note difference in scales of the y-axes.

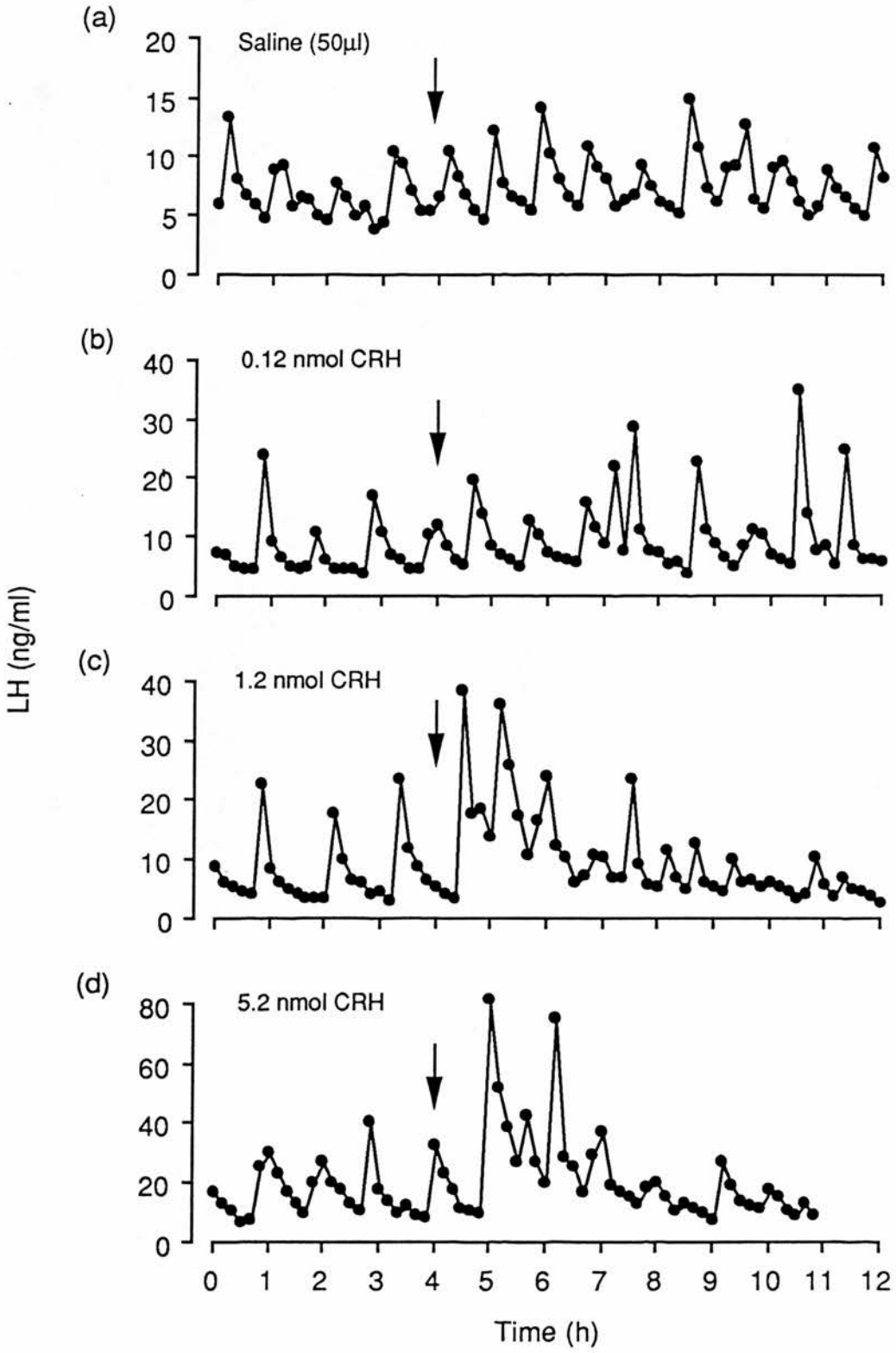
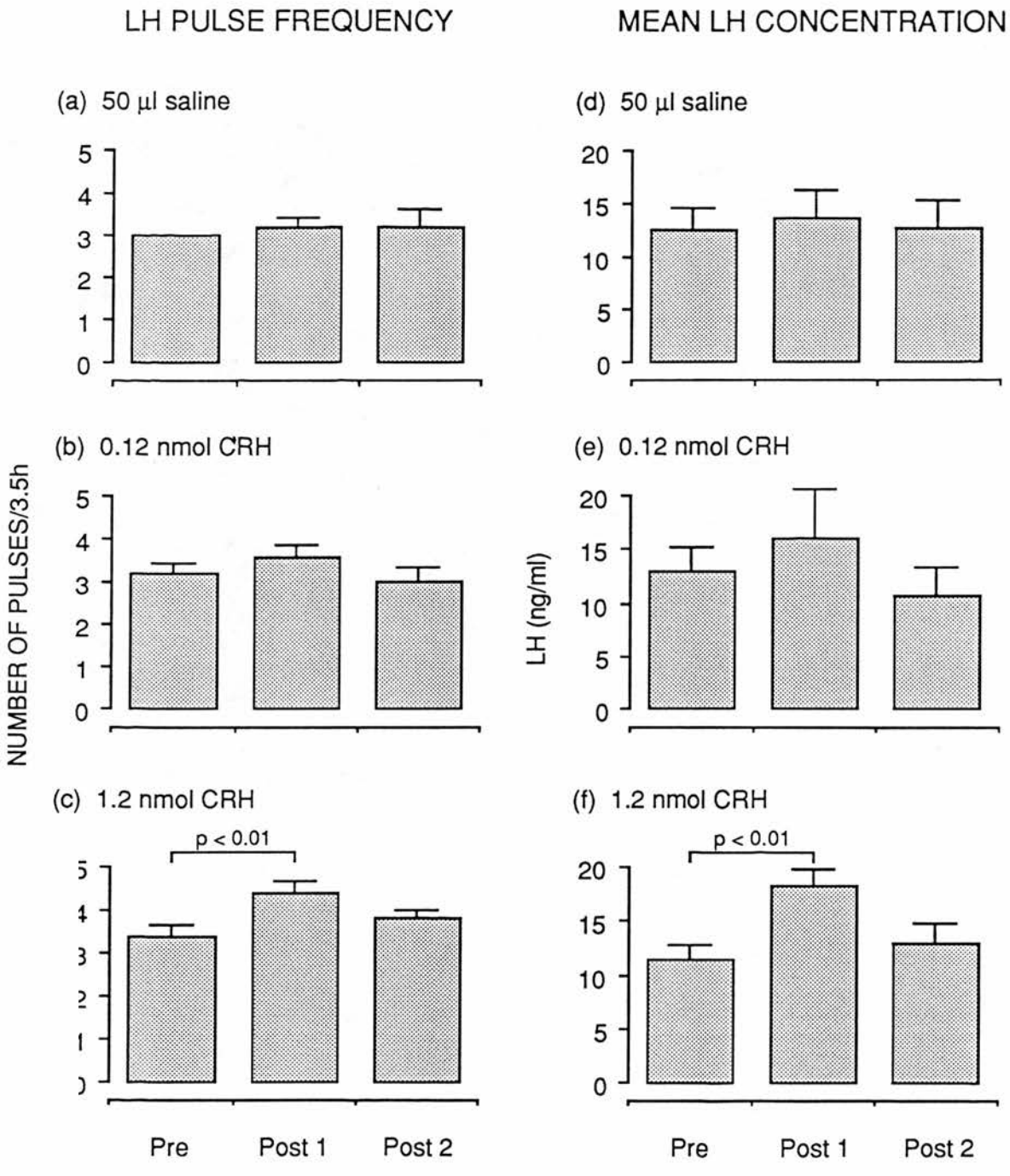
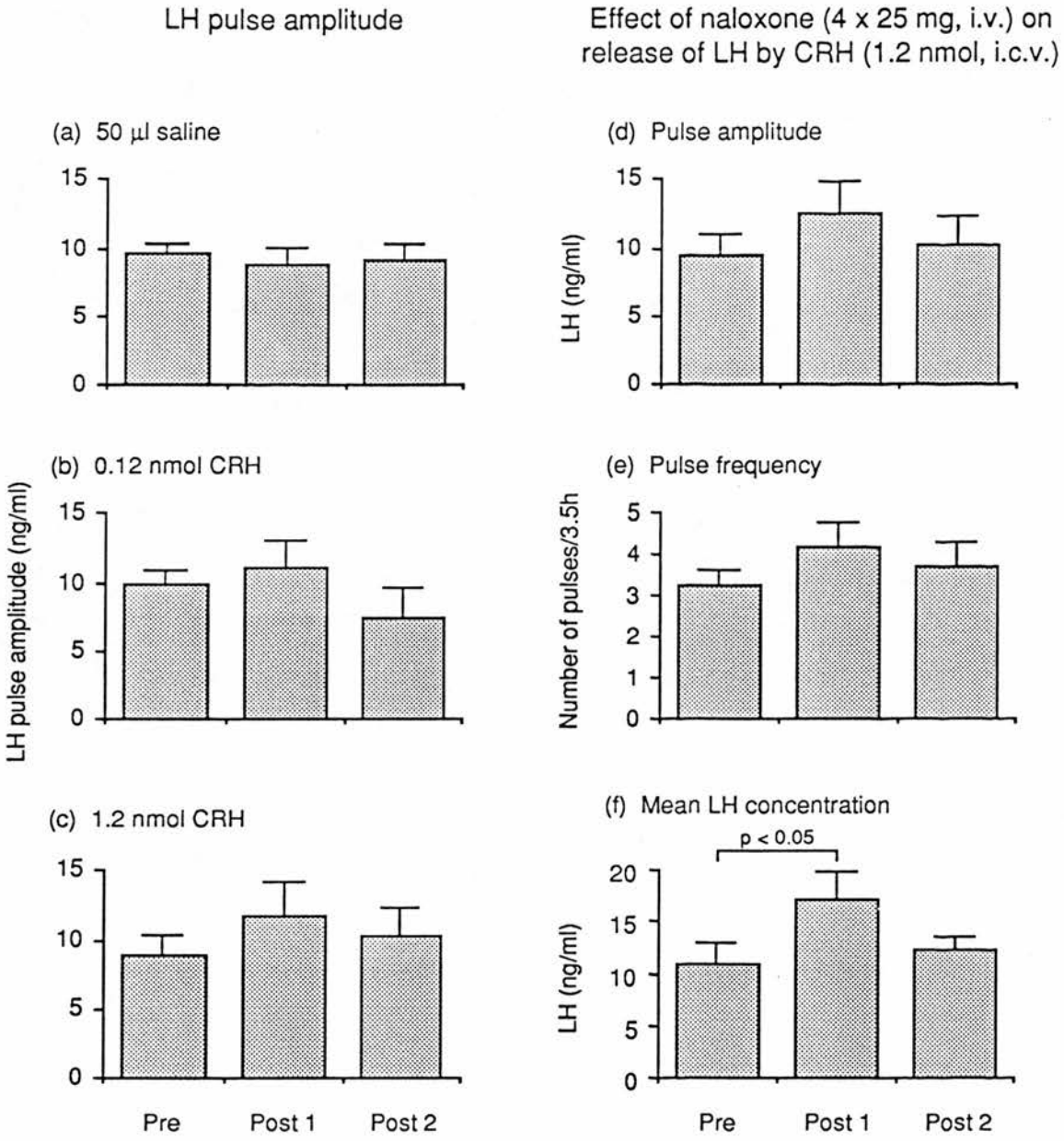

(a)
Saline (50µl)
20
15
10
5
0
(b)
0.12 nmol CRH
40
30
20
10
0
(c)
1.2 nmol CRH
40
30
20
10
0
(d)
5.2 nmol CRH
80
60
40
20
0
LH (ng/ml)
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (h)

Figure 7.2. Grouped data showing the effects of treatment with saline (50 μl) or 0.12 or 1.2 nmol CRH on (a–c) LH pulse frequency and (d–f) mean LH levels, before, during and after i.c.v. treatment. Values expressed as mean ± s.e.m. (n = 5). Levels of significance are shown. Note increase in mean LH levels as a result of an increase in pulse frequency, and that LH levels return to pre-injection levels in the post 2 time period. The increase in mean LH after 0.12 nmol CRH did not reach significance due to wide individual variation.


LH PULSE FREQUENCY
MEAN LH CONCENTRATION
(a) 50 µl saline
(b) 0.12 nmol CRH
(c) 1.2 nmol CRH
(d) 50 µl saline
(e) 0.12 nmol CRH
(f) 1.2 nmol CRH
NUMBER OF PULSES/3.5h
LH (ng/ml)
p < 0.01
p < 0.01
0
1
2
3
4
5
0
5
10
15
20
Pre
Post 1
Post 2



Addendum: Figure 7.2.1. (a–c) Effects of i.c.v. CRH on LH pulse amplitude. Note effect of CRH on pulse amplitude is not significant, due to large individual variation. (d–f) Effects of naloxone on CRH-induced increase in LH secretion. Note naloxone does not alter the ability of CRH to stimulate LH secretion. See Figure 7.2 for further details.

Figure 7.3. Effects of central injection of 50 µl saline or 1.2 nmol CRH on mean 20-min plasma concentrations of FSH. Time of injection (t = 4 h) shown by arrow. Values are expressed as the mean ± s.e.m. (n = 5). Central injection of 1.2 nmol CRH had no effect on plasma FSH levels.

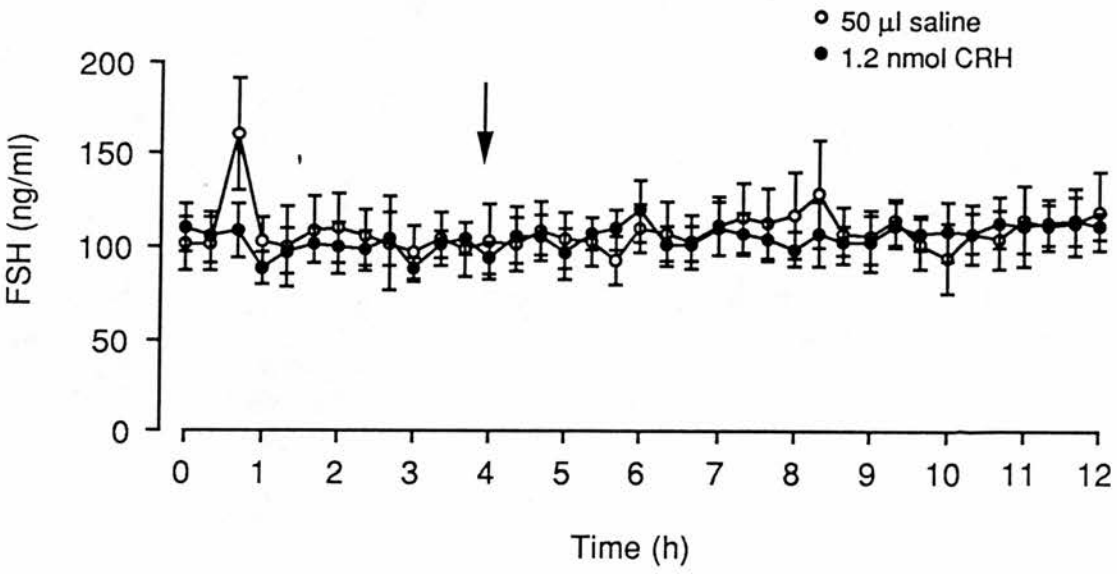
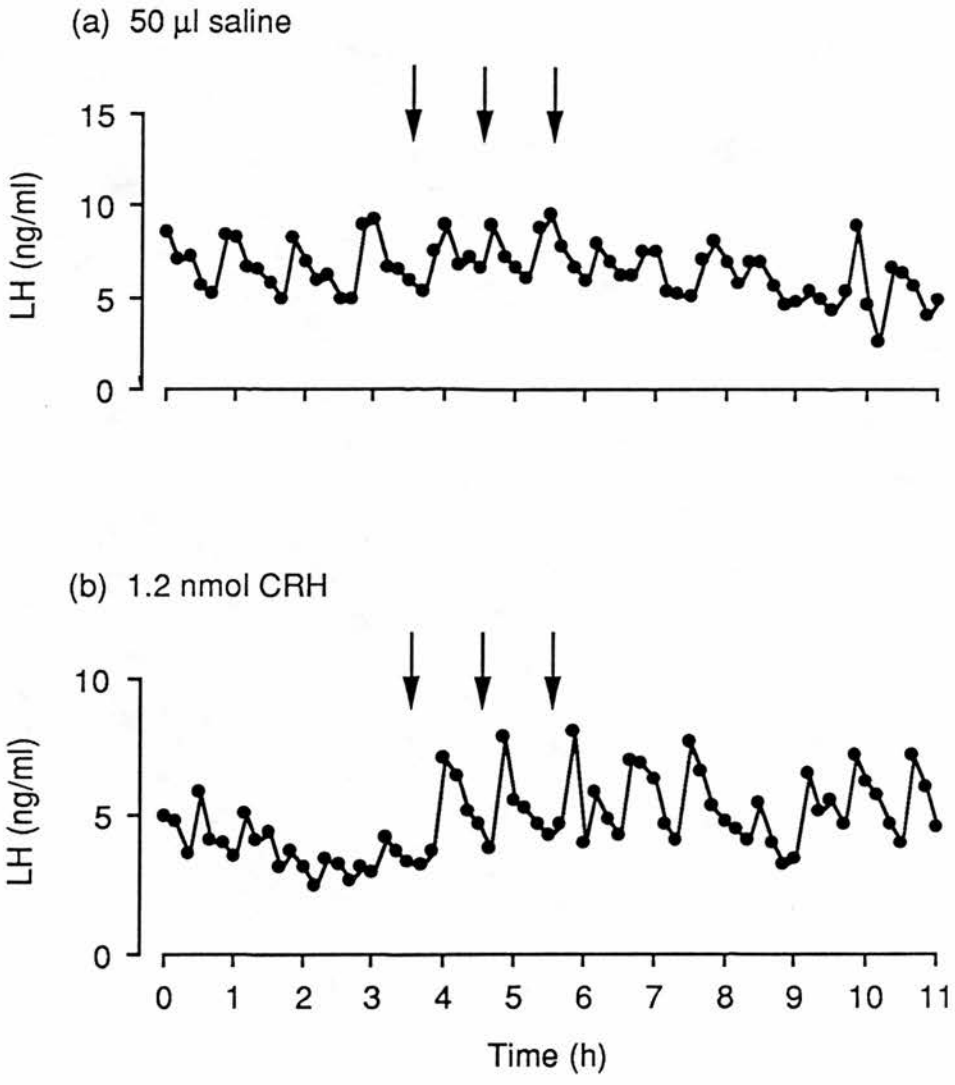

50 µl saline
1.2 nmol CRH
FSH (ng/ml)
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (h)

Figure 7.4. Concentrations of LH in 10-min plasma samples taken for 11 h in an individual ewe (No. R115), showing the effect of multiple injections of CRH on plasma LH. (a) three central injections of saline (50 μl) did not affect LH secretion. (b) triple injections of CRH (1.2 nmol) at t = 3.5, 4.5 and 5.5 h increased LH secretion. Arrows indicate times of injection.


(a) 50 µl saline
15
10
5
0
LH (ng/ml)
(b) 1.2 nmol CRH
10
5
0
LH (ng/ml)
0 1 2 3 4 5 6 7 8 9 10 11
Time (h)

injection of this opioid antagonist significantly increased mean LH levels, thus confirming our previous findings (Chapter 5).

The effects on mean plasma prolactin levels of i.c.v. injection of 1.2 nmol CRH and of 50 µl saline are shown in Figure 7.5a. Injection of saline into the third ventricle did not alter the normal pattern of prolactin secretion. In contrast, central administration of CRH evoked a marked stimulation of prolactin release from the pituitary gland which was evident within 20 min of the time of injection. This reached a maximum of approximately 3–4 times pre-injection levels after 80 min and had returned to baseline by 180 min. Figure 7.5b shows the effect of intravenous administration of naloxone on this stimulatory action of central CRH. In the presence of the opioid antagonist, CRH no longer evoked a large rise in plasma prolactin whilst naloxone alone did not alter basal prolactin release. The CRH-induced increase in plasma prolactin secretion seems to be dose-related, as shown in Figure 7.7a. The injection of a ten-fold lower dose of CRH (0.12 nmol) appeared to increase plasma prolactin levels compared with the control responses to saline, although this did not reach statistical significance. The higher dose of CRH increased prolactin secretion significantly, compared with both saline ($p < 0.01$) and the lower dose of CRH ($p < 0.05$). Figure 7.7a also demonstrates the effect of naloxone on the stimulation of prolactin by CRH. Naloxone reduced the CRH-induced stimulation, compared with CRH treatment alone ($p < 0.05$), whilst intravenous injection of naloxone alone was without effect.

tracerebroventricular injection of 1.2 nmol CRH also evoked a large rapid increase in the plasma levels of cortisol (Figure 7.6a) which l for longer than the effect on prolactin. In contrast to the prolactin nse, the central stimulatory effect of CRH on cortisol secretion was ected by the opioid antagonist naloxone (Figure 7.6b). Intravenous ion of naloxone alone did not alter basal cortisol release, compared the response to i.c.v. saline. The dose-related nature of the increase rtisol secretion is shown in Figure 7.7b: injection of both 0.12 nmol 0.05) and 1.2 nmol CRH ($p < 0.01$) increased plasma cortisol levels ficantly. Concomitant treatment with naloxone did not affect this lation ($p < 0.05$ compared with saline).

here was no significant difference between either the pre-injection ol values or the pre-injection prolactin values before each treatment not shown).

Figure 7.5, Effects of intracerebroventricular injection of CRH on mean plasma prolactin levels in samples taken every 20 min over the 12-h sampling period, showing that stimulation of prolactin by central CRH is opioid-mediated. (a) central injection of 1.2 nmol CRH causes a large and rapid rise in prolactin levels, whereas i.c.v. saline has no effect. ○ = saline, ● = CRH. (b) concomitant intravenous injection of naloxone (4 x 25 mg) abolishes the CRH-induced stimulation of prolactin, whereas i.v. naloxone alone is without effect. ○ = naloxone, ● = CRH + naloxone. Single arrows indicate times of central injection (t = 4 h), multiple arrows show times of i.v. naloxone injection (t = 4, 5.5, 7 and 8 h). Values are means ± s.e.m. (n = 5 for each treatment).

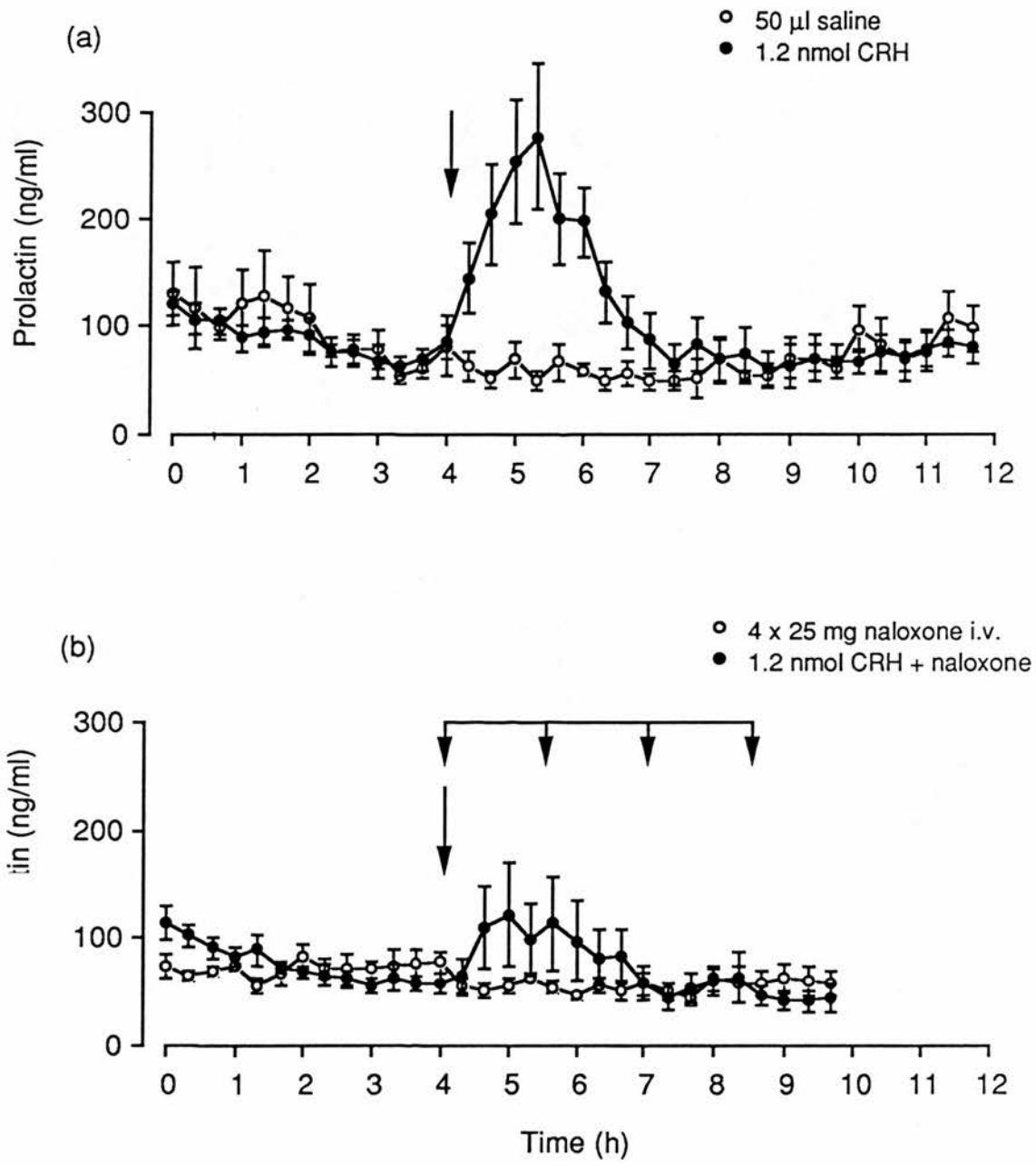

(a)
50 µl saline
1.2 nmol CRH
Prolactin (ng/ml)
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12
(b)
4 x 25 mg naloxone i.v.
1.2 nmol CRH + naloxone
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (h)

Figure 7.6. Effects of central injection of CRH on mean plasma cortisol levels in samples taken every 20 min over the 12-h sampling period, showing that the stimulation of cortisol secretion by central CRH is not mediated by opioids. (a) central injection of 1.2 nmol CRH causes a large and rapid rise in cortisol secretion, which lasts for longer than the rise in prolactin, whereas i.c.v. saline has no effect. ○ = saline, ● = CRH. (b) concomitant intravenous injection of naloxone (4 x 25 mg) does not diminish the increase in cortisol secretion caused by central CRH injection. Intravenous injection of naloxone alone has little effect on basal cortisol levels. ○ = naloxone, ● = CRH + naloxone. Single arrows indicate times of central injection (t = 4 h), multiple arrows show times of i.v. naloxone injection (t = 4, 5.5, 7 and 8 h). Values are means ± s.e.m. (n = 5 for each treatment).

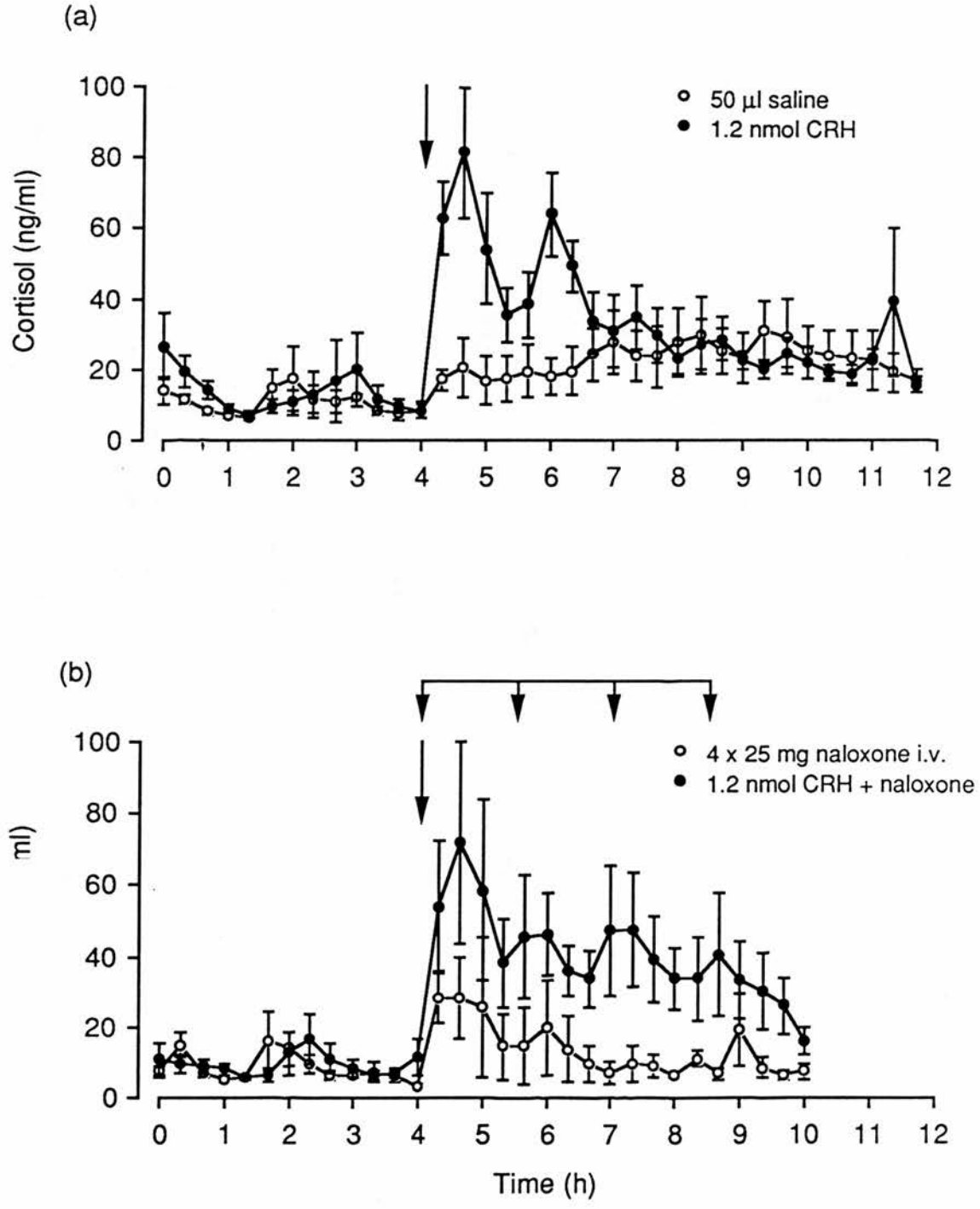
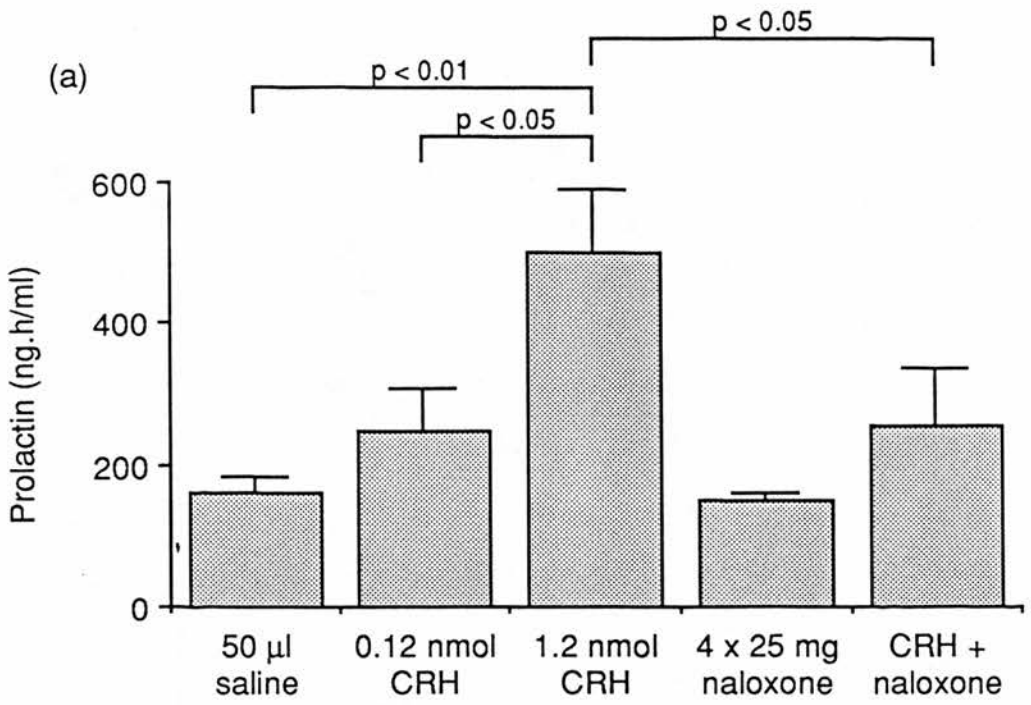
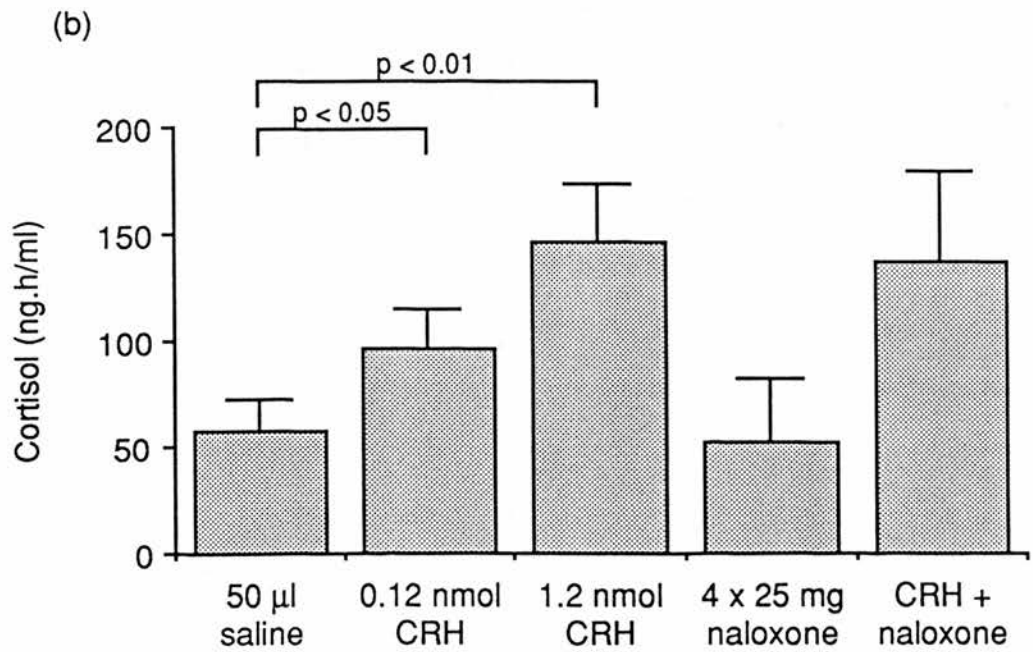

(a)
Cortisol (ng/ml)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
50 µl saline
1.2 nmol CRH
(b)
ml)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (h)
4 x 25 mg naloxone i.v.
1.2 nmol CRH + naloxone

Figure 7.7. Grouped effects of central administration of CRH on secretion of prolactin and cortisol and the effects of opioid antagonism on these responses. (a) shows the dose-related increase in prolactin secretion after central CRH and its abolition by naloxone. (b) shows cortisol secretion is also increased in a dose-related manner, but naloxone does not block this effect of CRH. Values are expressed as the mean area under the curve (± s.e.m.) in the 3 h following treatment. (n = 5 for each treatment). Plasma concentrations of prolactin and cortisol were not significantly different between treatments in the 3 h before injection.


(a)
p < 0.05
p < 0.01
p < 0.05
Prolactin (ng.h/ml)
600
400
200
0
50 µl saline
0.12 nmol CRH
1.2 nmol CRH
4 x 25 mg naloxone
CRH + naloxone


(b)
p < 0.01
p < 0.05
Cortisol (ng.h/ml)
200
150
100
50
0
50 µl saline
0.12 nmol CRH
1.2 nmol CRH
4 x 25 mg naloxone
CRH + naloxone

7.4. Discussion

Injection of CRH into the third cerebral ventricle of the ovariectomized ewe stimulated the release of LH and prolactin from the anterior pituitary gland, but had no effect on plasma concentrations of FSH. This lack of effect on FSH is an expected finding in view of the long half-life of FSH in ovariectomized ewes (Fry, Cahill, Cummins *et al.* 1987) and the fact that FSH secretion is normally unaffected by short-term alterations in the supply of LHRH to the pituitary gland (Fraser & McNeilly, 1983). The stimulatory effect of i.c.v. administration of CRH was dose-related in that the lower dose occasionally stimulated LH and prolactin release whereas the higher concentration consistently evoked the release of both hormones, that of prolactin by an opioid-dependent mechanism. The increase in mean LH concentration was due to a significant increase in LH pulse frequency, suggesting a hypothalamic site of action for CRH. Although pulse amplitude was often increased, there was too much individual variation for this to be statistically significant. Central administration of CRH also resulted in a significant increase in the plasma concentration of cortisol. Despite a postulated involvement of opioid peptides in regulating the hypothalamo-pituitary-adrenal axis (Monteleone, Maj, Iovino & Steardo, 1988), no evidence was obtained in these experiments to suggest that endogenous opioids are involved in

ıting the axis under either basal or stimulated conditions. In
ıst, naloxone stimulated LH release, thus confirming the findings
:ed in Chapter 5 that LHRH/LH secretion is under tonic inhibitory
l tone in long-term ovariectomized ewes.

e effect of central CRH on LHRH/LH secretion demonstrated by experiments in the sheep is the opposite to that described for the rat
r & Vale, 1984; Petraglia *et al.* 1987; Almeida *et al.* 1988) and monkey
r & Ferin, 1987; Gindoff & Ferin, 1987). In addition to the evidence
ɔ of an inhibitory effect of CRH on LH secretion, it has been well
nented that CRH suppresses the release of LHRH from rat
halamic slices *in vitro* (Gambacciani, Yen & Rasmussen, 1986;
ıarakis, Almeida & Herz, 1986*a*). Moreover, there is evidence that
hibitory effect of CRH in the rat is mediated through an activation of
;enous opioid pathways within the hypothalamus (Nikolarakis,
da & Herz, 1986*b*; Almeida *et al.* 1988; Nikolarakis *et al.* 1988).

It could be argued that the absence of an inhibitory effect of CRH in the sheep may be because an insufficient concentration of the peptide was injected into the brain. Although this possibility cannot be excluded completely, it is unlikely for the following reasons. Firstly, equimolar to higher than equimolar concentrations of CRH were used compared with those reported to be effective in the rat, and multiple injections of the peptide also did not suppress LH release. Secondly, the higher dose of CRH (1.2 nmol) was sufficient to stimulate prolactin and cortisol release. Alternatively, the presence of gonadal steroids such as oestrogen and progesterone may be required for central CRH to be able to inhibit LH secretion. However, this is unlikely as previous investigators have demonstrated that CRH inhibits LH release in ovariectomized rats and monkeys (Rivier & Vale, 1984; Gindoff & Ferin, 1987), as well as in intact female rats (Petraglia *et al.* 1987). An explanation of this discrepancy, therefore, may be that a species difference exists between sheep on the one hand and rats and monkeys on the other. In support of this view, the absence of an inhibitory effect of CRH on gonadotrophin secretion has been reported recently in the sheep following administration by two different routes. Using equimolar amounts of CRH to those used in the experiments described in this chapter, neither i.v. (Parrott, Robinson & Thornton, 1988) nor i.c.v. (Horton, Francis & Clarke, 1988) injection of CRH altered any characteristic of LH secretion. The reasons for the difference between this study and that of Horton *et al.* (1988) are unclear at

nt but seasonal factors governing the reproductive cycle of sheep may ence the response to centrally injected neuropeptides and transmitters.

different explanation for the stimulatory effect of central CRH ibed here is that the peptide preparation was contaminated and ined some factor with LH-releasing properties. An obvious date for this would be LHRH or its analogues. However, since al administration of LHRH inhibits LH secretion in sheep (see ter 4) this possibility is unlikely. In addition, the bioactivity (ACTH-ing property) of the batch of CRH used in this study was verified by igel Brooks using in-vivo and in-vitro models.

jection of CRH into the ventricular system of the sheep also evoked a release of prolactin into the peripheral circulation. This stimulatory was dose-related and could be prevented by the opioid antagonist one, suggesting that endogenous opioid peptides mediate the effect

of central CRH on prolactin secretion. An opioid-mediated stimulatory effect of intravenous CRH on prolactin secretion has recently been described in the monkey (Van Vugt, Webb & Reid, 1989). In addition, there is considerable evidence that opioid peptides themselves are involved in the stimulatory control of prolactin secretion (Dupont, Cusan, Labrie *et al.* 1977; Rivier, Vale, Ling *et al.* 1977; Gold, Redmond & Donabedian, 1979). Indeed, there is evidence that central opioid peptides are responsible for the increase in plasma prolactin observed in response to a number of stressful stimuli (Ferland, Kledzik, Cusan & Labrie, 1978; Van Vugt, Bruni & Meites, 1978). Since CRH stimulates prolactin release via opioid peptides, and as endogenous opioids have been implicated in the mediation of stress-induced prolactin secretion, it seems likely that the increase in plasma prolactin described here is a consequence of CRH acting as a neurotransmitter in a 'stress-responsive' pathway. In other words, CRH may serve as a central neurotransmitter (i.e., distinct from its ACTH-stimulating effect on the pituitary gland) involved in the integration of neuroendocrine responses to stressful stimuli. Although the experiments described in this chapter provide no *direct* evidence to support such a hypothesis, there is considerable evidence from other sources that CRH may mediate responses to stress by actions at the level of the CNS (Brown *et al.* 1982, 1985, 1986; Britton *et al.* 1982; Krahn *et al.* 1986; Kalin *et al.* 1988). The absence of a tonic opioidergic control of prolactin secretion is consistent with previous findings in non-lactating ewes (Horton, C[illegible]nins & Clarke, 1987).

though it seems probable that CRH-induced prolactin release is ıted through a central action, this does not appear to be the case for ffect of central CRH on cortisol secretion. The most likely nation for the stimulatory effect of i.c.v. administration of CRH on ɔl secretion is that when injected into the ventricular system, leakage H to the anterior pituitary gland occurred via the portal vessels and ɔy evoked the release of ACTH and cortisol. An alternative ɔility is that injection of CRH into the third ventricle augmented ;enous CRH release through the ultrashort-loop positive feedback n for CRH described by Ono, Bedran de Castro & McCann (1985). ver, such a hypothesis would require further testing using the direct ırement of endogenous CRH in the portal plasma of conscious . In contrast to their role in the action of central CRH on prolactin ion, endogenous opioids sensitive to blockade by naloxone are not

involved in the stimulatory effect of i.c.v. injection of CRH on cortisol secretion. Furthermore, in the ovariectomized sheep, like the rat (Buckingham & Cooper, 1986), opioid peptides do not exert a tonic influence (whether inhibitory or stimulatory) on the basal activity of the CRH-pituitary-adrenal axis as they do in humans (Monteleone *et al.* 1988).

If CRH does act as a neurotransmitter in a central 'stress-responsive' pathway then it may be possible to provide a further explanation as to why i.c.v. administration of CRH stimulated LH release in the sheep. Plasma concentrations of LH appear to be affected differently by acute as opposed to chronic stress. While chronic stress inhibits LH in intact and adrenalectomized rats (Gray *et al.* 1978; Taché *et al.* 1978), acute stress has been reported to stimulate LH release in both rats (Krulich, Hefco, Illner & Read, 1974; Euker, Meites & Riegle, 1975; Armario, Restrepo, Hidalgo & López-Calderón, 1987; Briski & Sylvester, 1988) and monkeys (Hayashi & Moberg, 1987), suggesting, perhaps, that the physiological response to stress is dependent upon the novelty, duration and intensity of the stimulus. In sheep, it has been reported that ovulation may be induced in anoestrus by the stress of shipping, a response that has been attributed to an increased output of gonadotrophins (Braden & Moule, 1964). In addition, the

increase in LH secretion in the ovariectomized ewe caused by the presence of a ram is blocked by the CRH antagonist $\alpha CRH_{(9-41)}$ (Dr Alasdair M. Naylor, unpublished observations). Furthermore, there is evidence in a recent report (López-Calderón, Gonzaléz-Quijano, Tresguerres & [illegible]avarreta, 1990) that the increase in plasma LH in response to acute

is due to an increase in hypothalamic LHRH secretion. It seems

, therefore, that the central injection of CRH activated a 'stress-

nsive' neural pathway(s) which manifested itself as an increase in

cretion of both LH and prolactin.

conclusion, the experiments described in this chapter demonstrate

n the sheep, central administration of CRH causes a dose-related

lation of LH, prolactin and cortisol release, that of prolactin by a

ss involving endogenous opioid peptides. These findings provide

:r evidence that CRH acts as a central transmitter in a neural

'ay(s) integrating endocrine responses to stress.

8. Neuroendocrine actions of centrally administered neuropeptide Y

8.1. Introduction

In the preceding chapters, interactions between several neuroendocrine control mechanisms were described and the hypothesis was developed that the hypothalamic releasing hormones could also act as central neurotransmitters. In addition to the releasing hormones, other hormonal peptides are known to modulate the primary neuroendocrine mechanisms regulating the secretion of hormones from the anterior pituitary gland.

One such factor is neuropeptide Y. This recently discovered peptide amide has been measured in very high concentrations in the hypothalamus (Allen, Adrian, Allen *et al.* 1983; Chronwall, DiMaggio, Massari *et al.* 1985). Neurones immunoreactive for NPY are abundant in the hypophysiotrophic areas of the brain (Everitt, Hökfelt, Terenius *et al.* 1984), NPY nerve terminals having been observed in close proximity to LHRH and CRH cell bodies in the OVLT and PVN, respectively (Bai,

no, Shiotani *et al.* 1985; Wahlestedt, Skagerberg, Ekman *et al.* 1987; Li & Pelletier, 1988).

PY has been shown *in vivo* and *in vitro* to modulate the activity of productive axis at the hypothalamic and pituitary levels. Depending e ovarian steroid environment, NPY can either stimulate or inhibit ecretion of LHRH and therefore of LH (Kalra & Crowley, 1984; ley, Tessel, O'Donohue *et al.* 1985; Kerkerian, Guy, Lefèvre & ier, 1985; McDonald, Lumpkin, Samson & McCann, 1985; Crowley & 1987; Khorram, Pau & Spies, 1987; Khorram, Pau & Spies, 1988; nald, Lumpkin & DePaolo, 1989).

PY appears also to have a stimulatory role on the hypothalamo-ary-adrenal axis, acting at the level of the hypothalamus. iments *in vivo* and *in vitro* have suggested that NPY can stimulate a ACTH levels, and that this is mediated by CRH in the PVN (Haas rge, 1987; Tsaragarakis, Rees, Besser & Grossman, 1989; Wahlestedt,

Skagerberg, Ekman *et al.* 1987; Härfstrand, Eneroth, Agnati & Fuxe, 1987; Inoue, Inui, Okita *et al.* 1989).

The studies in this chapter were therefore designed to examine the central actions of NPY on the hypothalamo-pituitary-ovarian and hypothalamo-pituitary-adrenal axes in the ewe and to investigate further the potential involvement of ovarian steroids in the modulation of these axes by NPY. Specifically, the effect of intracerebroventricular administration of NPY on LH and cortisol secretion was investigated in ovariectomized ewes, with or without oestradiol-containing implants, and in intact ewes in the follicular and luteal phases of the oestrous cycle.

8.2 Experimental design

For the experiments described in this chapter, three different groups of animals were used; i.e., ovariectomized (OVX) ewes, ovariectomized ewes implanted with oestradiol (OVX/E_2), and intact cycling ewes.

8.2.1. Ovariectomized ewes

During August–September 1988, six Scottish Blackface ewes were ovariectomized and implanted with a stainless steel cannula directed towards the third ventricle as described previously.

Two to three months later, the animals were housed for experiment in ...idual pens in the University of Edinburgh Marshall Building, near ...burgh, from October–November 1988, under artificial lighting ...tions corresponding to natural daylength. They were fed concen... pellets once a day and hay and water were available *ad libitum.*

Ovariectomized ewes implanted with oestradiol

... Scottish Blackface ewes were ovariectomized in October 1988 and ...nted one month later with a stainless steel cannula directed towards ...ird cerebral ventricle.

... least one week prior to experimentation, the animals were ...nted subcutaneously with oestradiol in silastic tubing according to ...ethod of Karsch, Dierschke, Weick *et al.* (1973), modified only to the ... of using smaller tubing. Briefly, 1.5-cm lengths of silastic medical-... tubing (outside diameter 3.5 mm, inside diameter 2 mm, Dow... ng Medical, Midland, MI, USA), were packed with crystalline 17β-

oestradiol (Sigma, Poole, Dorset) and sealed with silastic elastomer (Dow-Corning). This was done away from the laboratory and sheep-facility premises to prevent possible contamination of other studies. In order to avoid an initial transient burst of oestradiol release from the capsule after placement, the implants were soaked overnight in water and then stored in 70% ethanol for approximately 30 min before insertion. Two capsules were implanted via a 1–2 cm incision, made under lignocaine local anaesthesia, in the shaved axillary skin of each ewe. They were inserted subcutaneously and pushed 5–6 cm away from the incision site. The wound was then sutured. Since assays sufficiently sensitive to estimate peripheral levels of oestradiol in ewes were not available, it was not possible to measure the plasma concentrations achieved. However, these or similar implants have been shown previously to produce physiological levels of oestradiol in the circulation (Dr Jon D. Curlewis, personal communication; see also Goodman, Bittman, Foster & Karsch, 1981; Moenter, Caraty & Karsch, 1990).

For the experiment, the animals were housed and fed as described above (Section 8.2.1) from December 1988–January 1989.

8.2.3. Blood sampling and data analysis in OVX and OVX/E_2 ewes

On the day before each sampling period, a jugular venous cannula was inserted into each ewe and kept patent with heparinized saline. Blood samples (3 ml) were collected at 10-min intervals for 10–11 h from 08.00 h

e day of each experiment. After an initial 4-h control period, the
ıls received a single central injection of human NPY (see Section 3.4)
ɜ of two doses, 0.15 nmol (0.65 μg) or 1.5 nmol (6.5 μg), or of 50 μl
:. Each ewe received each treatment and thus served as her own
ɔl.

ɜsma concentrations of LH were measured in samples taken every 10
ɪnd cortisol levels in half-hourly samples. For the analysis of the LH
the sampling period was divided into three 3-h windows
ɜenting time periods before (pre), during (post 1) and after (post 2) the
ıl injections. Mean cortisol concentrations in the 3 h after injection
compared with those in the preceding 3 h.

Intact ewes

Finn x Dorset ewes (37–46.5 kg) were implanted with a stainless steel

guide cannula directed towards the third ventricle in July 1989. Four months later, during the breeding season (November 1989), the animals' oestrous cycles were synchronized prior to the start of experiments in December.

The initial cycle synchrony was achieved by withdrawal of progestagen-impregnated intravaginal sponges (Veramix, 600 mg medroxyprogesterone acetate, Upjohn Animal Health, Crawley, Sussex) 12 days after their insertion. All ewes displayed behavioural oestrus within 48 h as detected by a vasectomized ram wearing a coloured wax marker crayon strapped to his chest (oestrus = day 0).

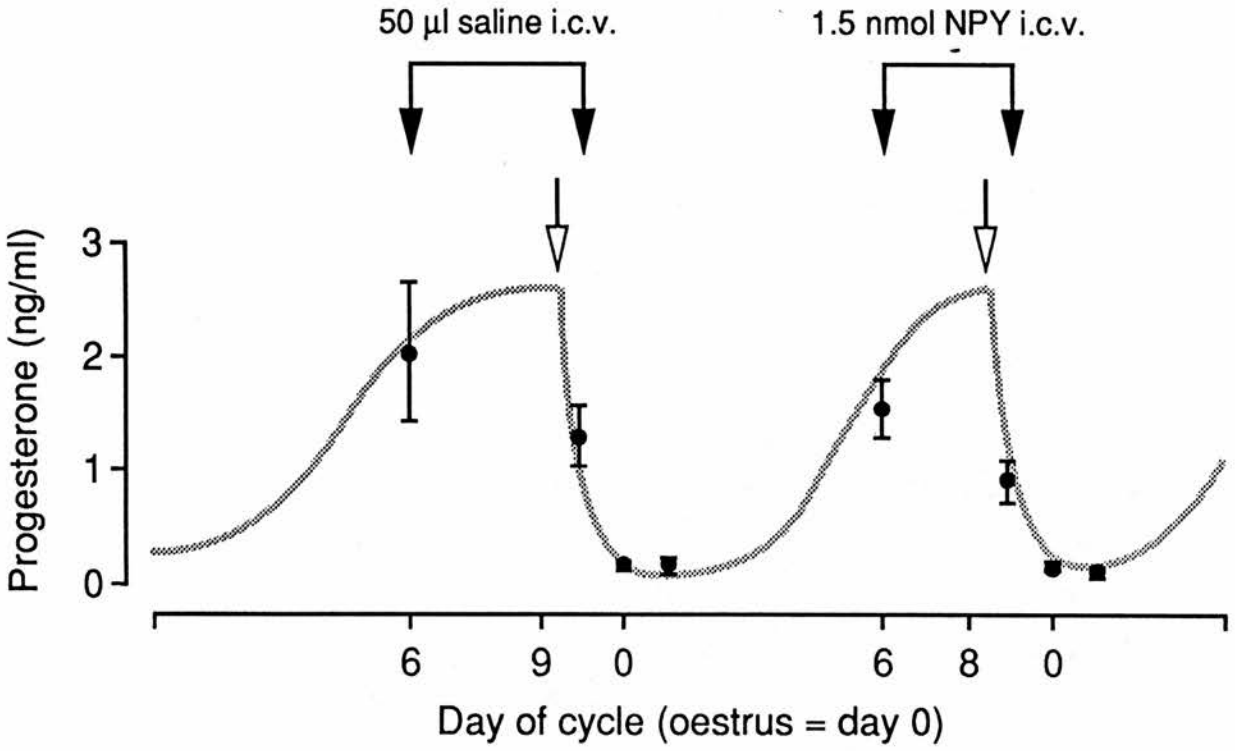
On day 6 of the cycle, i.e. during the luteal phase, blood samples (1.5 ml) were taken every 10 min from each animal for 10 h from 09.00 h to 19.00 h, via a jugular venous cannula inserted the previous day. After a 3-h control period, all animals received an injection of 50 µl saline into the third ventricle. At the end of the 10-h sampling period, the cannulae were removed and the animals allowed to recover. The ewes were re-cannulated on the afternoon of day 9 of the same oestrous cycle. Luteolysis was then induced at 23.00 h with an intramuscular injection of 100 µg cloprostenol, a potent analogue of prostaglandin $F_{2\alpha}$ (0.4 ml Estrumate, Coopers Animal Health Care, Crewe, Cheshire). The next day, the sheep were sampled every 10 min for 10 h from 09.00 h, i.e. starting at 10 h after prostaglandin administration, during the follicular phase of the second cycle. Blood samples were then taken hourly for a further 60 h,

07.00 h on the fourth day of sampling. After the first 3 h of blood
ing, each animal again received an injection of 50 µl saline.

ree days later, the animals were cannulated again for a 10-h period of
sampling every 10 min on the following day (day 6). Sampling
started at 09.00 h and after a 3-h control period, the animals received
nol NPY, injected into the third ventricle. As before, at the end of
)-h sampling period, the cannulae were removed and the animals
ed to recover. The ewes were then re-cannulated on the afternoon of
luteolysis was induced at 23.00 h with prostaglandin $F_{2\alpha}$ analogue as
and, the next day, the sheep were sampled every 10 min for 10 h
09.00 h. Blood samples were taken hourly for a further 60 h, until
h on the fourth day of sampling. After the first 3 h of blood
ing, each animal received an injection of 1.5 nmol NPY.

ure 8.1 shows the progesterone levels measured in samples taken
the experiments on the intact cycling ewes to illustrate the design

Figure 8.1. Progesterone concentrations in intact cycling ewes, illustrating the timing of i.c.v. saline/NPY injections (closed arrows) relative to the oestrous cycle(s). ● = mean plasma levels of progesterone in Finn x Dorset ewes during the experiment ± s.e.m. (n = 5). Shaded line is diagrammatic representation of progesterone concentration throughout the cycle (after Wallace, Martin & McNeilly, 1988). Injections of cloprostenol to induce luteolysis (see text) shown by open arrows.


50 µl saline i.c.v.
1.5 nmol NPY i.c.v.
Progesterone (ng/ml)
3
2
1
0
6
9
0
6
8
0
Day of cycle (oestrus = day 0)

in relation to stage of the cycle(s). Details of the progesterone assay are given below.

Plasma concentrations of LH were measured in samples taken every 10 min for the first 10 h of sampling, and then in samples taken every hour for the remainder of the sampling period. Changes in LH secretion were analysed as for the OVX and OVX/E_2 ewes (Section 8.2.3). The onset of the LH surge was defined as the time after cloprostenol when LH levels were first raised above 10.0 ng NIH-LH-S23 equiv/ml as part of a sustained rise in LH concentration. Cortisol was measured in half-hourly samples for the first 10 h. As there was large inter-animal variation in the pre-injection levels of cortisol, baseline secretion was calculated as the mean of the concentrations measured in the 3 h preceding central injection. The secretion of cortisol in the remaining 6 h was then expressed as a percentage of this. For analysis, the data were condensed by calculating the mean cortisol levels in each hour after i.c.v. injection.

8.2.5. Progesterone assay

Plasma progesterone was measured by non-extraction radioimmunoassay as follows. Briefly, samples were assayed in duplicate in a volume of 50 µl. Pooled plasma (50 µl) from ovariectomized sheep was added to 100 µl of each standard (5.0–1 000 pg/100 µl, Sigma) and 50 µl sample was added to 100 µl buffer (phosphate citrate buffer containing 0.1% gelatin; pH 6.0). The anti-progesterone antibody (S361) used in the assay was raised in a
by Dr R.J. Scaramuzzi (Prospect, NSW, Australia) against progest-
-11α-hemisuccinate conjugated to BSA. It was added at a dilution of
)00 in a volume of 100 µl. Radiolabelled progesterone was prepared
ı Swanston from a progesterone-11α-glucuronide-tyramine conjugate
the chloramine-T technique. 100 µl ^{125}I-progesterone (15 000 cpm)
dded to each tube and the assay incubated overnight at 4 °C for 24 h.
r was diluted in phosphate citrate buffer without gelatin, but with
8-anilino-1-naphthalene-sulphonic acid/ml buffer. 1 ml 25% poly-
ene glycol (PEG) was added and tubes were then centrifuged at 3 000
2 110 *g*) for 30 min at 4 °C. The centrifuge buckets were then placed
e and the supernatant aspirated, leaving a white precipitate which
hen counted as previously. All samples were included in the same
and intra-assay variation was less than 8%.

8.3 Results

We were unable to demonstrate any effect on LH secretion of the administration of exogenous NPY into the third cerebral ventricle, in any of the three groups of animals. Mean LH levels, LH pulse amplitude and LH pulse frequency were unchanged.

Figure 8.2a–c shows the lack of effect on LH secretion of centrally injected saline and of two doses of NPY (0.15 and 1.5 nmol) in representative ovariectomized ewes. Similarly, injection of NPY at these doses was without effect in ovariectomized ewes implanted with oestradiol, as shown in Figure 8.2d–f. The lack of effect of NPY treatment on mean LH levels before, during and after injection in both OVX ewes and OVX/E_2 ewes is shown in Figure 8.3 for each group of animals.

A cursory glance at Figure 8.3b suggests that the lower dose of NPY, and also the control treatment with saline, may possibly have had an effect on the secretion of LH in these OVX/E_2 animals. Taking the group as a whole, this was not the case. However, in several OVX/E_2 animals treated with either saline or the lower dose (0.15 nmol) of NPY we observed a gradual suppression of LH secretion starting at the time of injection. In these animals, there was also a slow increase in plasma cortisol concentrations. This was surprising, as in our other studies we found little effect of i.c.v. saline on cortisol secretion and no effect on LH (see ters 6 and 7). A possible explanation for these observations is the ving. These animals had not been used for experiment for some ns and it may be that, on introducing the needle to administer the nents, we may have caused tissue damage around the end of the cannula. If this were the case, it could have resulted in the lation of prostaglandin (Rudy, Williams & Yaksh, 1977) and/or eukin-1 production (Rivier, Chizzonite & Vale, 1989), with quent activation of the hypothalamo-pituitary-adrenal (HPA) axis. could be reflected in the increased secretion of cortisol and, mably, of ACTH. Based on the results of Chapter 6, this may have ed in a suppression of LH secretion to account for our observations. te this, the animals have been included in the analysis, as we have rect proof that the i.c.v. injections were in fact compromised in such

.

gure 8.4 illustrates that i.c.v. injection of 1.5 nmol NPY also had no

Figure 8.2. Concentrations of LH in 10-min blood samples over an 11-h sampling period in (a–c) ovariectomized ewes and (d–f) ovariectomized ewes implanted with oestradiol, showing lack of effect of centrally-administered NPY at two doses on LH secretion. LH profiles are from several representative ewes treated i.c.v. with (a, d) 50 μl saline, (b, e) 0.15 nmol and (c, f) 1.5 nmol NPY. Time of injection is indicated by arrow (t = 4 h). LH concentration expressed in terms of NIH-LH-S23.

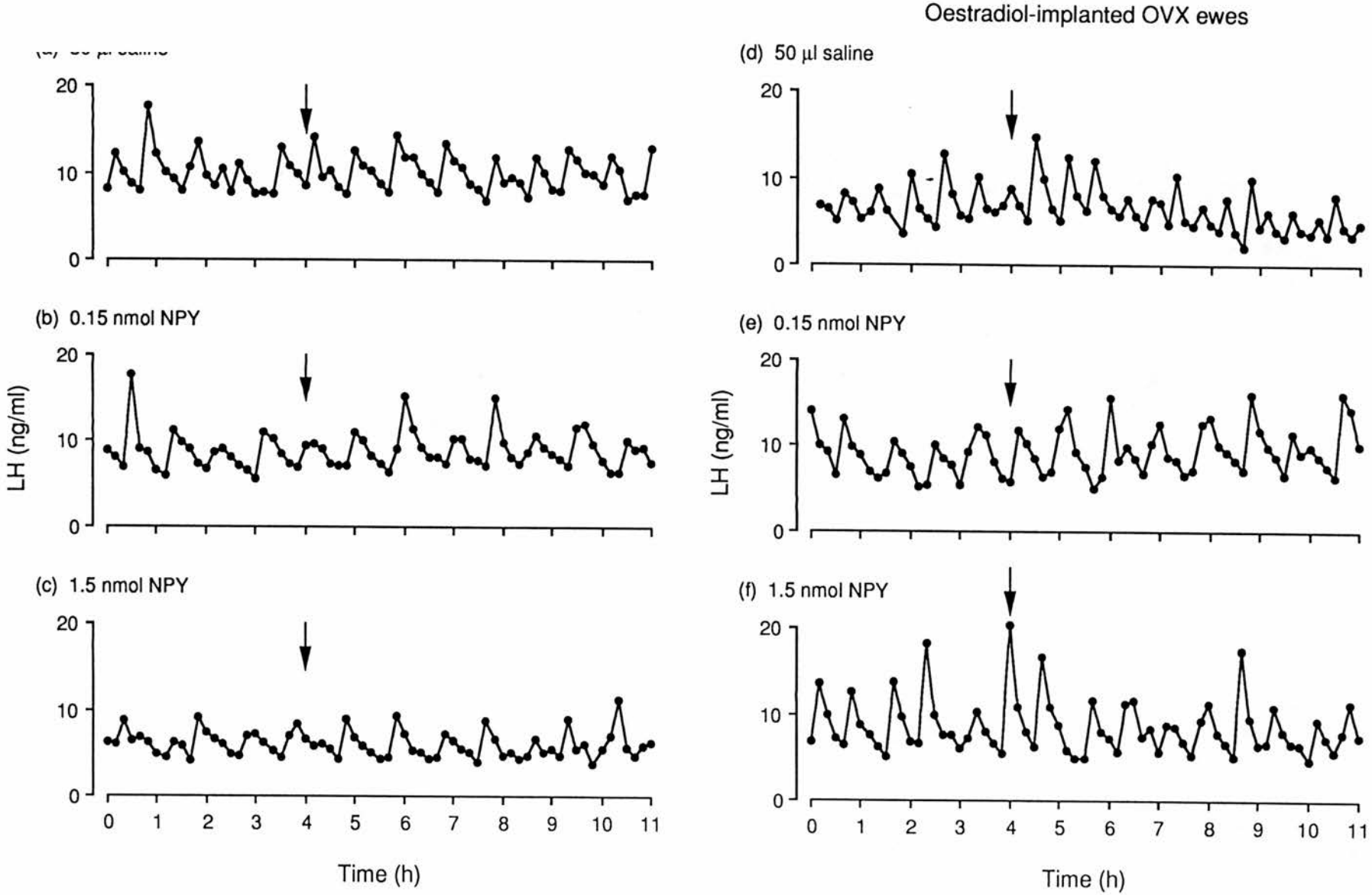
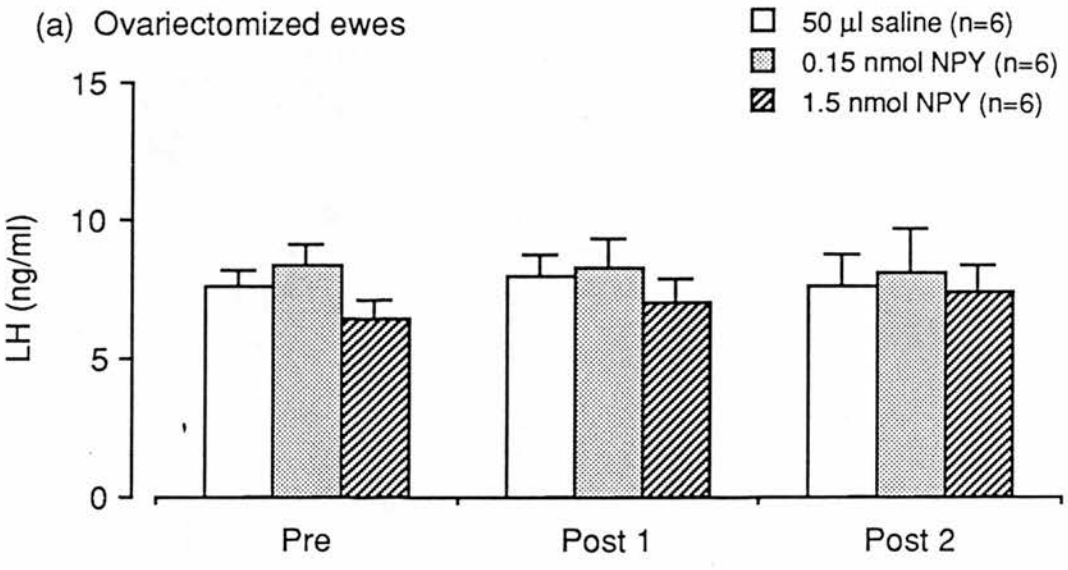

Oestradiol-implanted OVX ewes
(d) 50 µl saline
(b) 0.15 nmol NPY
(e) 0.15 nmol NPY
(c) 1.5 nmol NPY
(f) 1.5 nmol NPY
LH (ng/ml)
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
Time (h)

Figure 8.3. Grouped data showing the effects of i.c.v. treatment with 50 µl saline or 0.15 or 1.5 nmol NPY on mean LH levels in 3-h time periods before (Pre), during (Post 1) and after (Post 2) central injection in (a) ovariectomized ewes (n = 6) and (b) ovariectomized ewes implanted with oestradiol (n = 7). Values expressed as mean ± s.e.m. There was no effect of NPY at either dose on LH secretion. LH concentrations in terms of NIH-LH-S23.


(a) Ovariectomized ewes
50 µl saline (n=6)
0.15 nmol NPY (n=6)
1.5 nmol NPY (n=6)
LH (ng/ml)
15
10
5
0
Pre
Post 1
Post 2

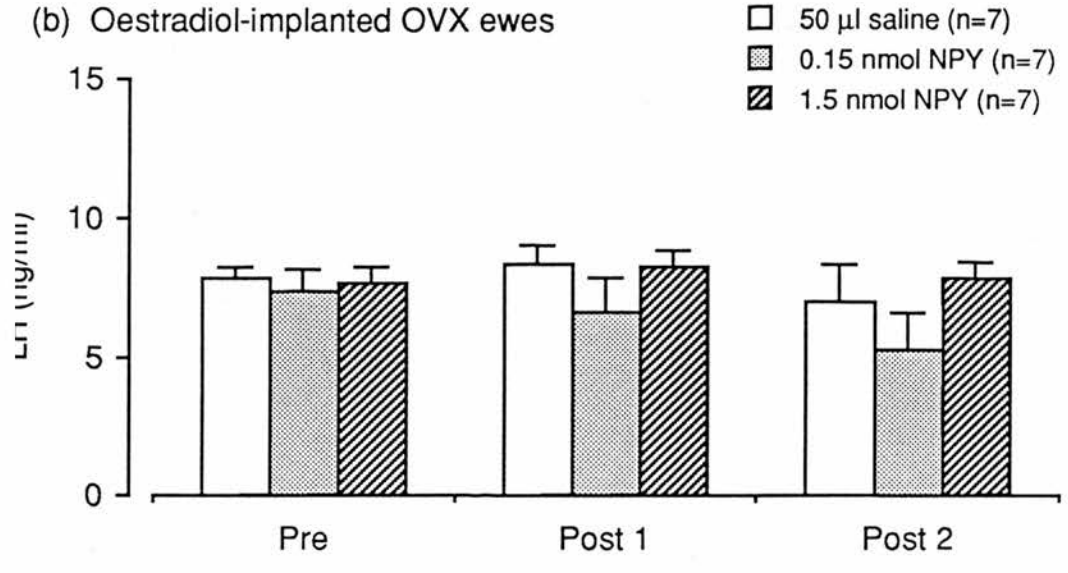
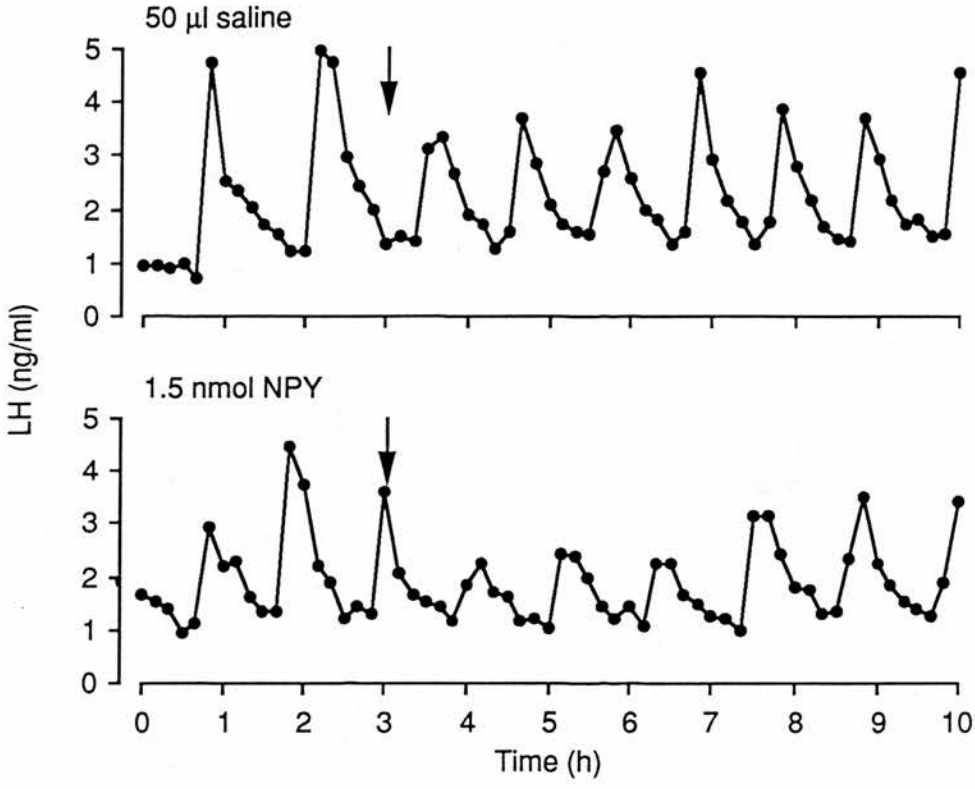
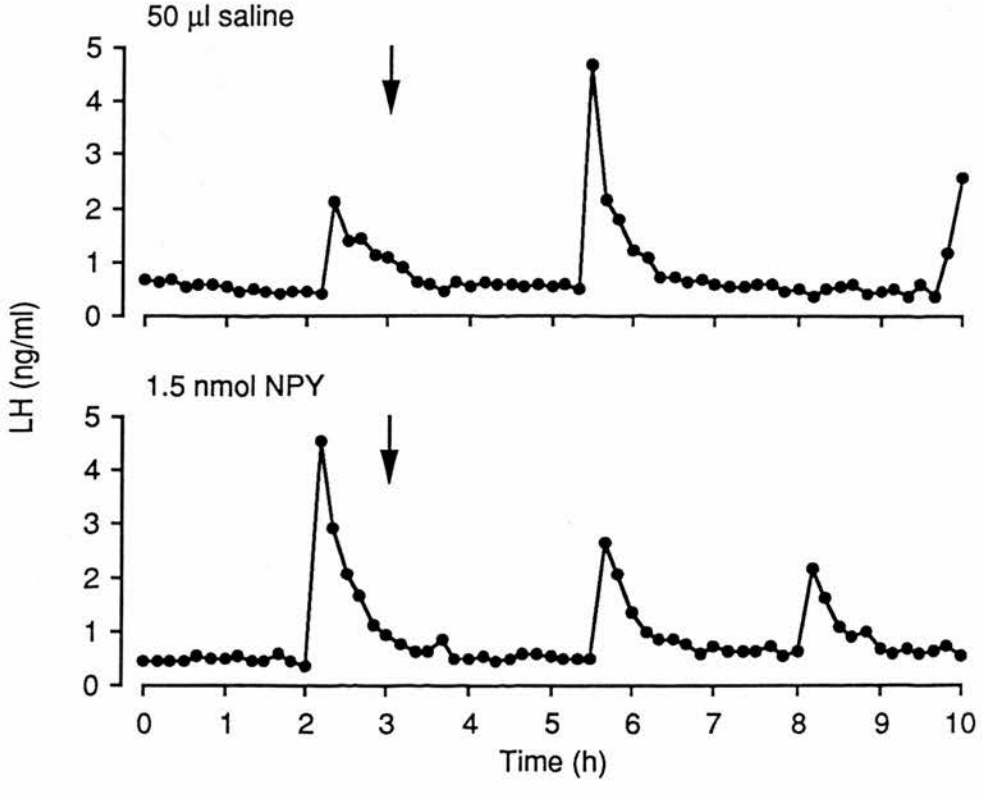

(b) Oestradiol-implanted OVX ewes
50 µl saline (n=7)
0.15 nmol NPY (n=7)
1.5 nmol NPY (n=7)
15
10
5
0
Pre
Post 1
Post 2

Figure 8.4. Injection of 1.5 nmol NPY into the third cerebral ventricle has no effect on LH secretion in the intact ewe, whether given in (a) the follicular phase, or (b) the luteal phase. Concentrations of LH are shown in 10-min samples over a 10-h sampling period for two ewes. Control and treated data from the same animal. Time of injection (t = 3 h) as shown by the arrow. LH levels expressed in terms of NIH-LH-S23.

(a) Follicular phase



(b) Luteal phase

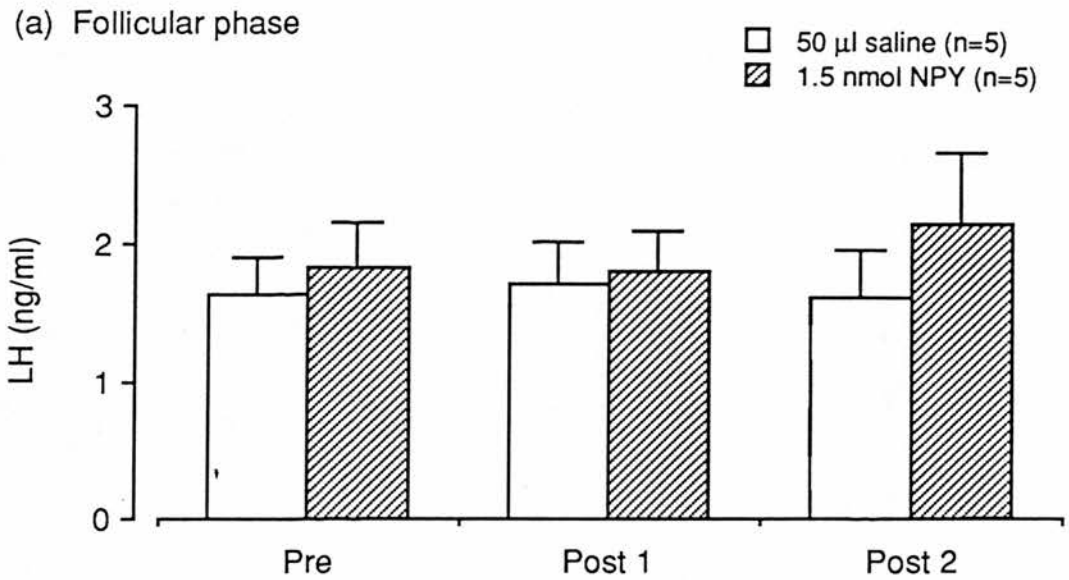


effect on LH secretion in the intact ewe, whether injected in the follicular (Figure 8.4a) or the luteal (Figure 8.4b) phase of the oestrous cycle. There was no effect of NPY on mean LH levels before, during or after NPY injection in either phase of the cycle (Figure 8.5). In addition, NPY treatment in the follicular phase had no effect on the timing (time to peak; NPY, 51.8 ± 2.22 h vs. saline, 53.8 ± 1.78 h; n = 5 in each group) or characteristics (peak height; NPY, 104.4 ± 18.58 ng/ml vs. saline, 141.7 ± 20.77 ng/ml; n = 5 per group) of the pre-ovulatory LH surge.

The effects of central injection of NPY on plasma cortisol concentrations in ewes of the various groups are shown in Figures 8.6–8.9. Central administration of 1.5 nmol NPY resulted in increased secretion of cortisol in both OVX/E_2 ewes (Figure 8.6f) and, though less consistently, OVX ewes (Figure 8.6c). Injection of 0.15 nmol NPY produced variable responses in the two groups. There was little or no effect on cortisol secretion in most OVX ewes (Figure 8.6b), though several animals responded with a large stimulation of cortisol secretion. The majority of OVX/E_2 ewes responded to the lower dose with an increased secretion of cortisol (Figure 8.6e).

The effects of central administration of saline or NPY on mean cortisol levels in the 3 h after injection in OVX and OVX/E_2 ewes are shown in Figures 8.7a and 8.7b for each dose. Central injection of 1.5 nmol NPY appeared to result in a modest increase in cortisol secretion in OVX ewes (Figure 8.7a), as did the injection of 0.15 nmol NPY. However, the
ıse to the lower dose was equivocal as indicated by the large standard
shown in the figure. There was no effect of NPY in the majority of
ınimals, but several responded with a large increase in activity of the
ıl axis. In OVX/E_2 ewes the responses to NPY were more consistent
e 8.7b). Although the effect of the lower dose was very variable,
animals responded with a large increase in cortisol secretion. The
ıse to the 1.5 nmol dose of NPY in OVX/E_2 ewes was much more
tent and indeed reached statistical significance ($p < 0.05$). Figure 8.7c
ates the change in mean cortisol secretion effected by 1.5 nmol NPY
s group of animals during the 11-h sampling period. The mean
a levels of cortisol in half-hourly samples are shown.
e effects of saline and central NPY injection on cortisol secretion in
dual intact ewes in the follicular and luteal phases of the cycle are
ı in Figures 8.8a–c and 8.8d–f respectively. NPY administration
g the follicular phase stimulated the secretion of cortisol

Figure 8.5. Grouped effects of NPY treatment on mean LH levels before, during and after injection in (a) the follicular (n = 5) and (b) the luteal phase (n = 5) of the oestrous cycle. Central injection of 50 µl saline or of 1.5 nmol NPY had no effect on mean LH concentrations. Bars represent the mean concentrations of LH (± s.e.m.) in the 3-h time periods before (Pre), during (Post 1) and after (Post 2) i.c.v. injection. LH levels expressed as NIH-LH-S23.


(a) Follicular phase
50 µl saline (n=5)
1.5 nmol NPY (n=5)
LH (ng/ml)
3
2
1
0
Pre
Post 1
Post 2

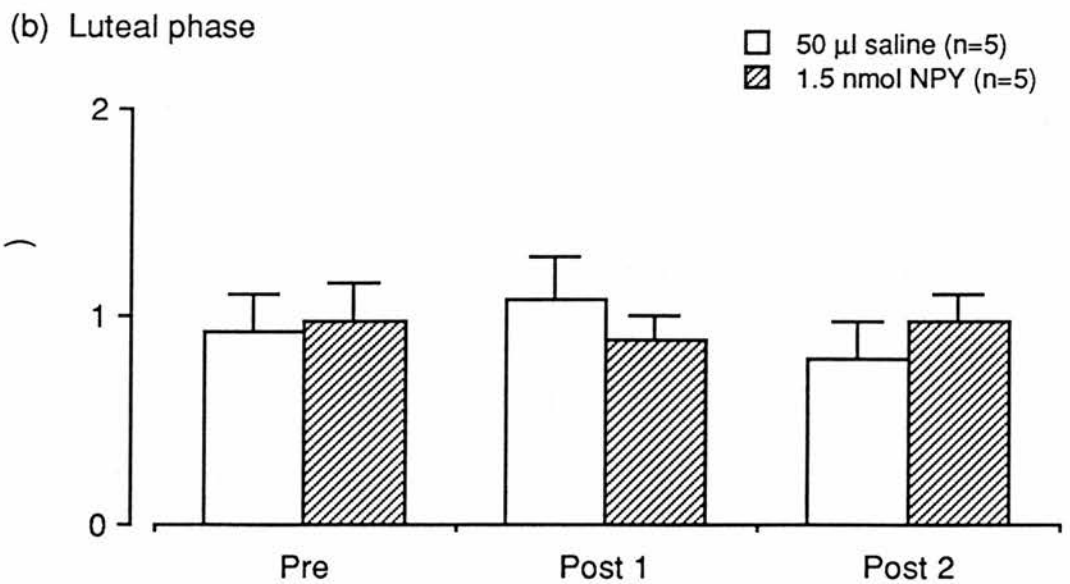

(b) Luteal phase
50 µl saline (n=5)
1.5 nmol NPY (n=5)
2
1
0
Pre
Post 1
Post 2

Figure 8.6. Plasma levels of cortisol in half-hourly samples taken over the 11-h period, showing the effects of i.c.v. injection of saline or NPY (0.15 or 1.5 nmol) on cortisol secretion in (a–c) representative ovariectomized ewes (n = 6) and (d–f) ovariectomized ewes implanted with oestradiol. Third ventricular injection of 1.5 nmol NPY (c, f) resulted in increased secretion of cortisol in both groups. (e) injection of 0.15 nmol NPY increased cortisol secretion in the majority of OVX/E_2 ewes, but (b) had little effect in most OVX ewes. Time of injection (t = 3 h) shown by arrows.

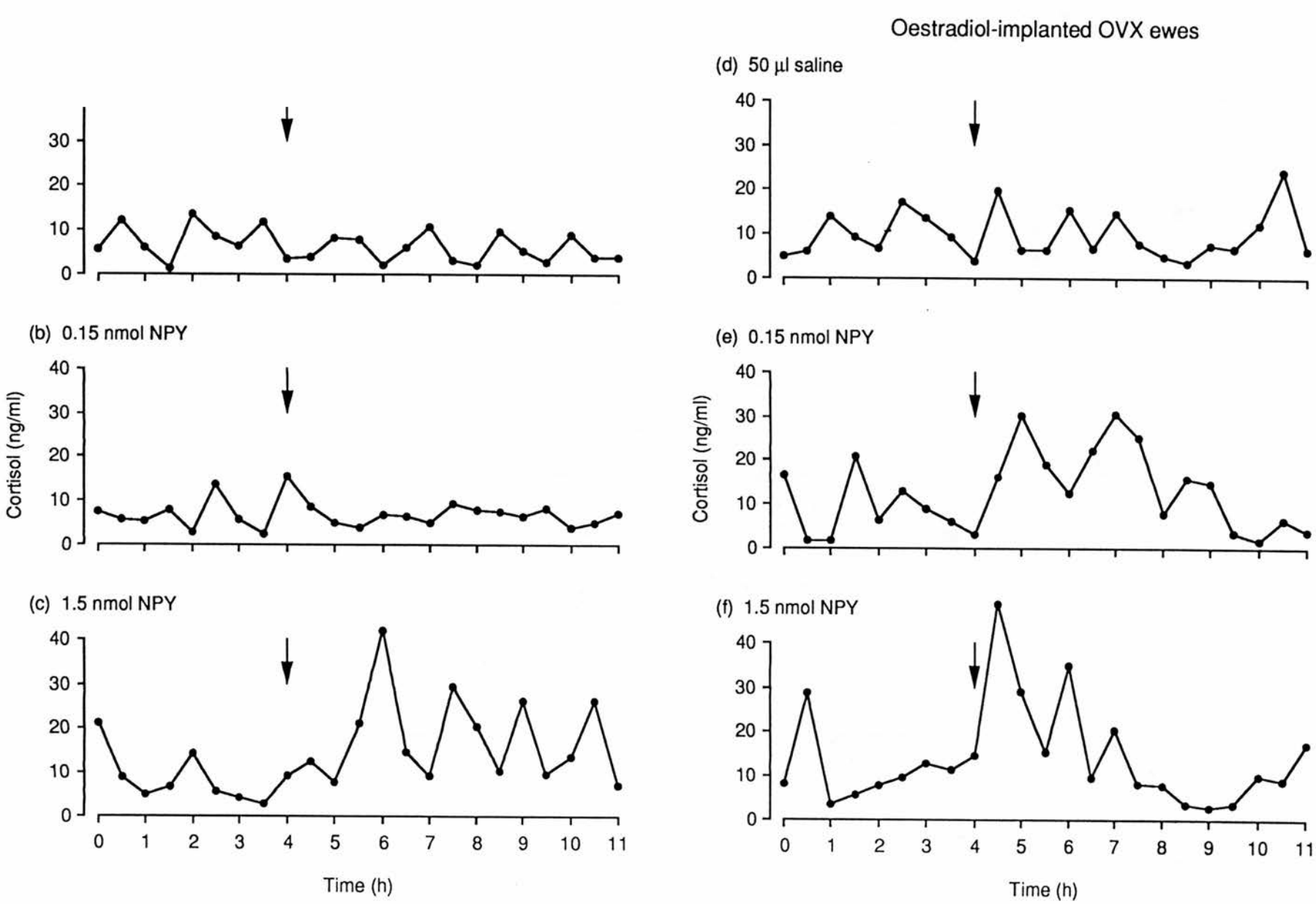

Oestradiol-implanted OVX ewes
(d) 50 µl saline
(b) 0.15 nmol NPY
(e) 0.15 nmol NPY
(c) 1.5 nmol NPY
(f) 1.5 nmol NPY
Cortisol (ng/ml)
Cortisol (ng/ml)
0
10
20
30
40
0 1 2 3 4 5 6 7 8 9 10 11
Time (h)
Time (h)

Figure 8.7. Effects of the central injection of NPY on mean plasma concentrations of cortisol in (a) ovariectomized ewes (n = 6) and (b, c) ovariectomized ewes implanted with oestradiol (n = 7). (a, b) effects of i.c.v. saline and two doses of NPY (0.15 and 1.5 nmol) on mean cortisol levels in the 3 h after central injection, compared with the 3 h before. Data shown are mean ± s.e.m. NPY stimulated cortisol secretion in both OVX and OVX/E_2 ewes, though this only reached significance at the higher dose in the OVX/E_2 group ($p < 0.05$). (c) effect of i.c.v. injection of 1.5 nmol NPY on mean half-hourly levels of cortisol in OVX/E_2 ewes (n = 7). Time of injection (t = 4 h) as shown by arrow.

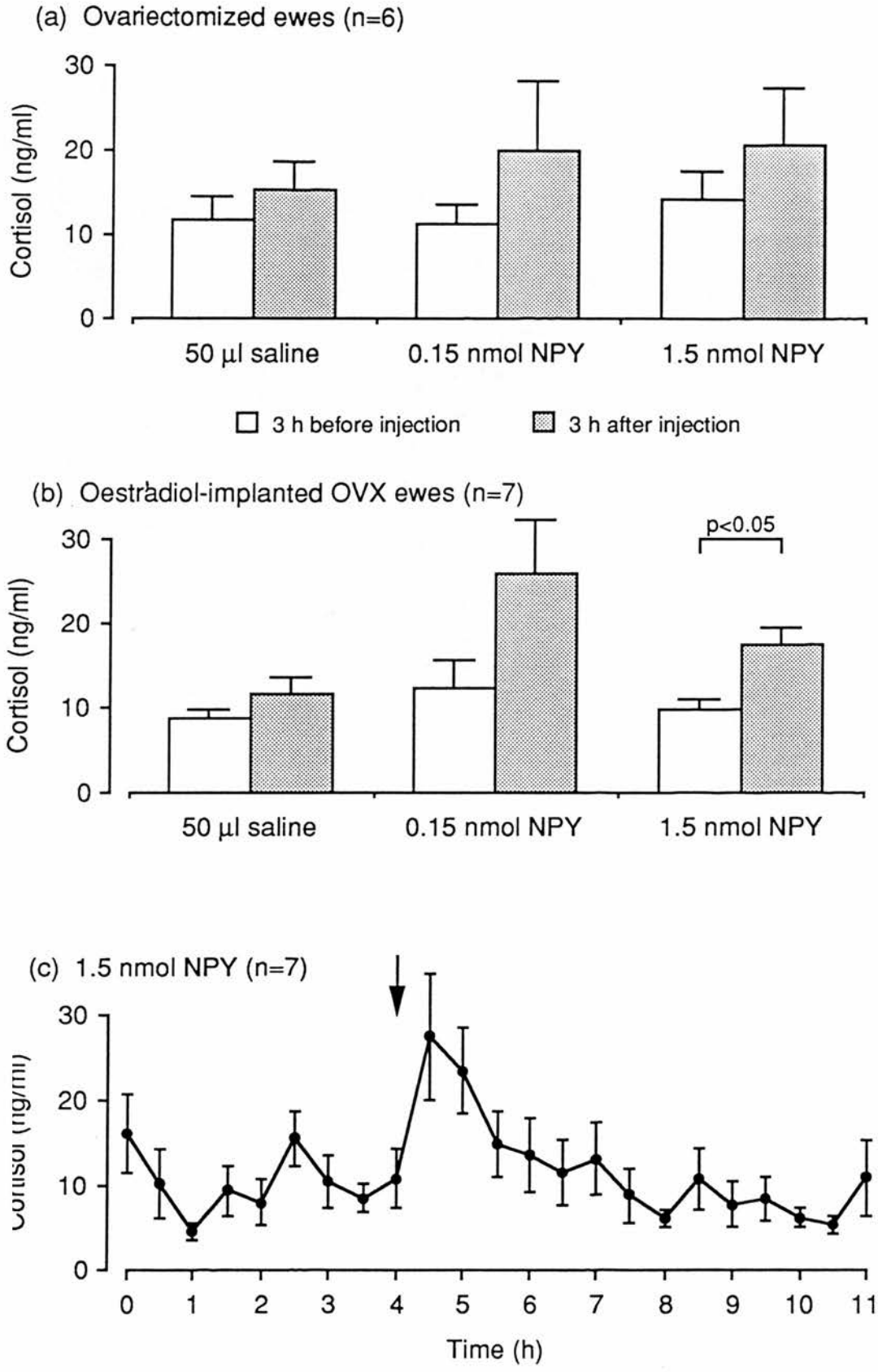
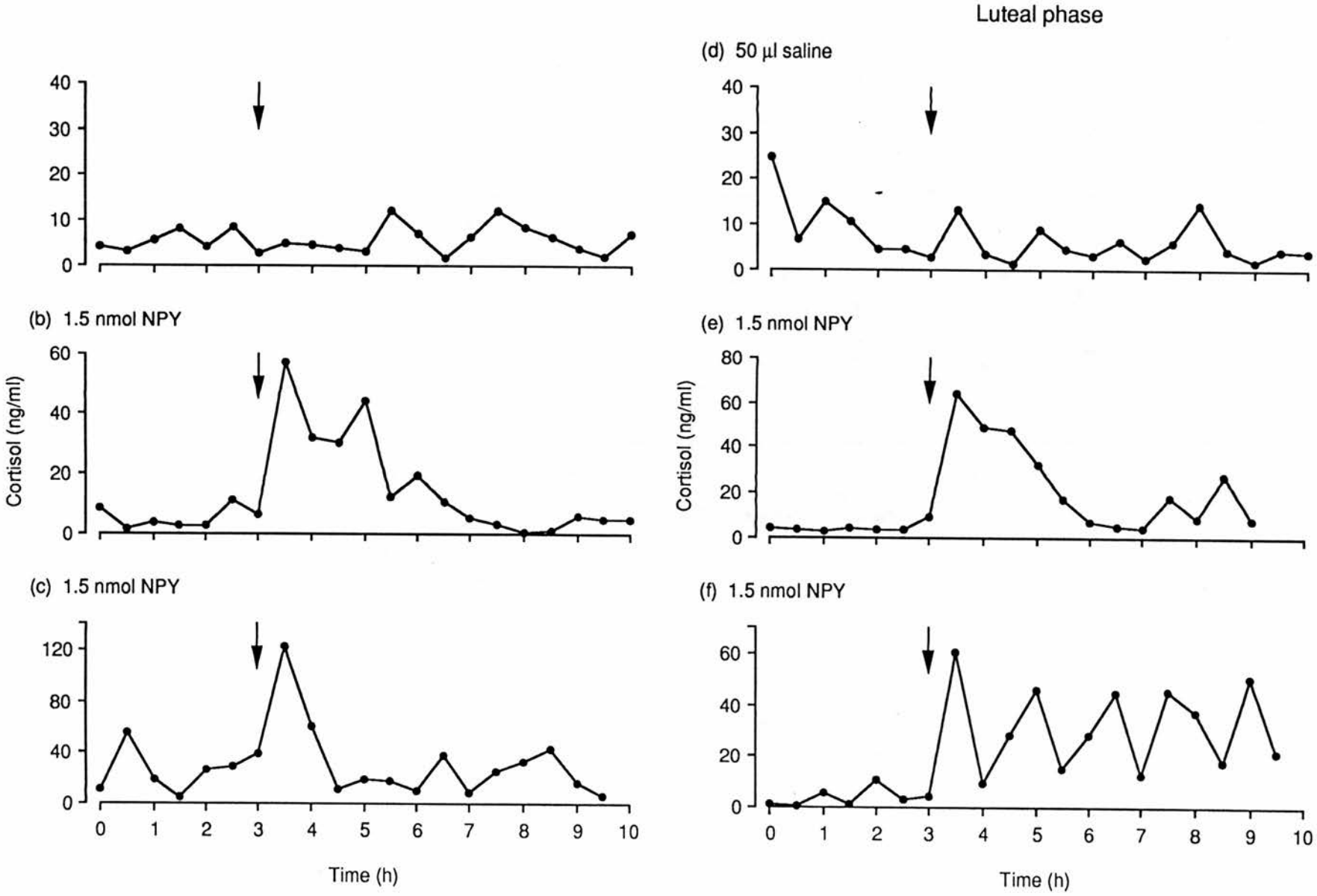

(a) Ovariectomized ewes (n=6)
Cortisol (ng/ml)
30
20
10
0
50 µl saline
0.15 nmol NPY
1.5 nmol NPY
3 h before injection
3 h after injection
(b) Oestradiol-implanted OVX ewes (n=7)
p<0.05
(c) 1.5 nmol NPY (n=7)
0 1 2 3 4 5 6 7 8 9 10 11
Time (h)

Figure 8.8 Effects of i.c.v. injection of 50 µl saline or 1.5 nmol NPY on plasma levels of cortisol in half-hourly blood samples over a 10-h period in individual ewes in (a–c) the follicular phase and (d–f) the luteal phase of the oestrous cycle. Note different scales of the y-axes. Injection of 1.5 nmol NPY in the follicular phase resulted in a variable increase in cortisol secretion. However, injection of this dose in the luteal phase stimulated cortisol secretion consistently in all animals. (b, c) cortisol profiles of two animals responsive to NPY in the follicular phase. (e, f) representative responses to NPY in the luteal phase.


Luteal phase
(d) 50 µl saline
(b) 1.5 nmol NPY
(e) 1.5 nmol NPY
(c) 1.5 nmol NPY
(f) 1.5 nmol NPY
Cortisol (ng/ml)
Cortisol (ng/ml)
Time (h)
Time (h)

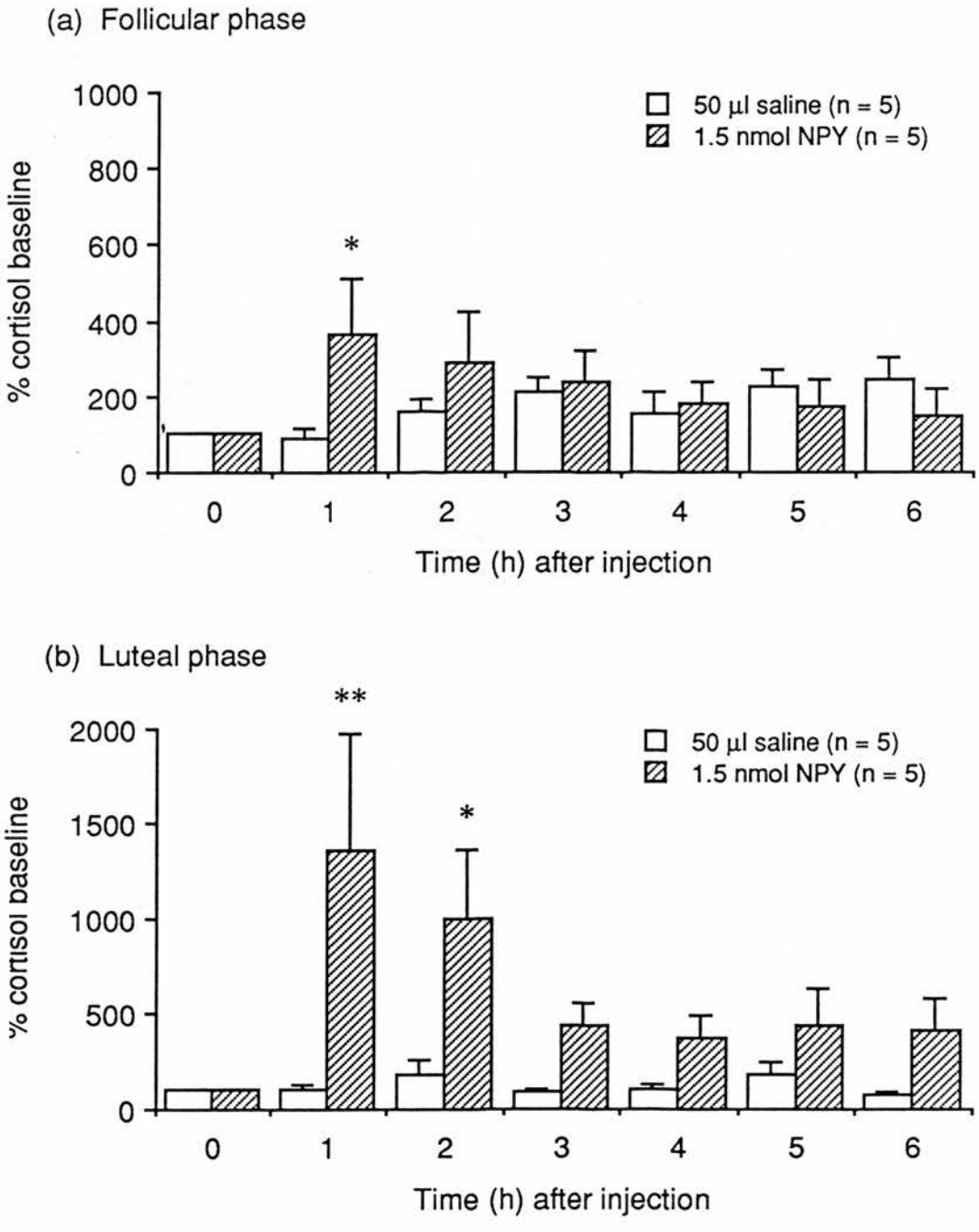
considerably in some animals though only modestly in others. The effects of 1.5 nmol NPY injected i.c.v. on plasma concentrations of cortisol in two of the animals that showed responses during the follicular phase are shown Figures 8.8b and 8.8c. The response shown in Figure 8.8c was unusually large, but short-lived. In contrast, NPY treatment at this dose in the luteal phase stimulated cortisol secretion consistently, as shown by the representative responses in Figures 8.8e and 8.8f. Interestingly, two out of five animals' responses were as in Figure 8.8e, whereas the other three were as shown in Figure 8.8f.

The effects of i.c.v. NPY on mean cortisol levels during the follicular and luteal phases in the group of animals are shown in Figures 8.9a and 8.9b respectively. As the baseline levels of cortisol were very variable between animals, the results after injection were first expressed in terms of the percentage of each animal's baseline in the 3 h prior to injection. The means of these normalized results are presented in the figure. Central injection of 1.5 nmol NPY stimulated significantly the secretion of cortisol relative to baseline in both the follicular (Figure 8.9.a) and the luteal (Figure 8.9b) phases of the oestrous cycle, the response to NPY being particularly robust in the luteal phase.

8.4 Discussion

Administration of neuropeptide Y into the third ventricle of the ewe had
ect on any aspect of LH secretion at the doses tested, whether in the
ctomized ewe, the ovariectomized ewe implanted with oestradiol,
he intact ewe in the follicular or the luteal phase.
contrast, NPY administration stimulated the HPA axis in the intact
)VX/E_2 animal, though only inconsistently in the OVX ewe. The
ation of cortisol secretion was modest, though significant, in the
E_2 ewe and in the follicular phase, but profound in the luteal phase
oestrous cycle.
e lack of effect of NPY on LH secretion in the studies reported in this
r is in marked contrast to data obtained in other species, where
l administration of NPY showed clear stimulatory and inhibitory
(Kalra & Crowley, 1984; Kerkerian, Guy, Lefèvre & Pelletier, 1985;
nald, Lumpkin, Samson & McCann, 1985; Khorram, Pau & Spies,
There are several possible explanations for this, most of which can
counted. It is unlikely that the doses of NPY used were too low for

Figure 8.9. Grouped mean cortisol concentrations in the 6 h after i.c.v. injection of 1.5 nmol NPY in (a) the follicular phase (n = 5) and (b) the luteal phase (n = 5), expressed relative to the baseline secretion of cortisol in the 3 h preceding injection. Note different scales of y-axes. Central injection of NPY stimulated cortisol secretion relative to baseline in both the follicular and luteal phases. However, the stimulatory effect was much greater in the luteal phase. Data shownaremean ± s.e.m. * $p < 0.05$, ** $p < 0.01$, compared with the response to saline at the same time after injection.


(a) Follicular phase
% cortisol baseline
1000
800
600
400
200
0
50 µl saline (n = 5)
1.5 nmol NPY (n = 5)
*
0
1
2
3
4
5
6
Time (h) after injection
(b) Luteal phase
% cortisol baseline
2000
1500
1000
500
0
**
*
50 µl saline (n = 5)
1.5 nmol NPY (n = 5)
0
1
2
3
4
5
6
Time (h) after injection

several reasons. Firstly, the doses employed resulted not only in a robust stimulation of the adrenal axis in the OVX/E_2 ewe and in the luteal phase, but also caused a noticeable, but unquantifiable, increase in feeding behaviour. This effect on appetite was not such a remarkable phenomenon as that reported for the rat, however, where NPY injected into the third ventricle (Clark, Kalra, Crowley & Kalra, 1984) or the PVN (Stanley & Leibowitz, 1984; 1985) evoked a potent feeding response. Secondly, the central administration of NPY to sheep at doses 10- to 100-fold greater than those used in this study have been shown by Clarke's group to be without effect on LH secretion (Dr Iain J. Clarke, personal communication). It is also unlikely that the lack of effect on LH secretion is due to an insufficient quantity of the peptide reaching the active sites relevant to an effect on the reproductive axis. The hypophysiotrophic areas of the sheep hypothalamus are located immediately adjacent to the third ventricle, and there is direct access to some of these, such as the OVLT (McKinley, Denton, Leventer *et al.* 1983). It remains a possibility, however, that centrally administered NPY acts on LHRH secretion in opposite directions via different pathways, resulting in no net observable effect when measured in terms of LH secretion.

Another plausible explanation for the inability of NPY to affect the LHRH/LH axis is that the structure of the exogenous, human, NPY used in the study compromised its physiological capacity to influence LHRH secretion. At the time the experiments were performed, the structure of

NPY was not known. However, it has since been characterized and to differ from human NPY by only two amino acids, at positions 10 7, and from porcine NPY by only one amino acid, at position 10 d, Agerberth, Mutt & Jörnvall, 1989). These substitutions are rvative (aspartate for glutamate at position 10, and leucine for onine at position 17) and it seems highly unlikely that these minor es are capable of altering the tertiary structure of the molecule in ı way as to alter dramatically its biological activity. In any case, the ıat central administration of human NPY has such a profound effect ɜ adrenal axis illustrates that it does possess biological activity with l to the sheep.

e most likely explanation for the lack of effect of NPY on LHRH and cretion, therefore, is that a true species difference exists between the d the rabbit on one hand, and the sheep, and also the cow, on the

In addition to our results *in vivo*, and those of Clarke's group,

there is evidence from experiments *in vitro* that NPY is without effect on pituitary LH secretion in these species. In contrast to data obtained primarily in the rat, NPY has no modulatory influence on either basal or LHRH-stimulated LH release from cultured ovine pituitary cells (Brooks, Graham & Naylor, 1990) and nor does it modulate the release of LH from bovine pituitary cells in culture (Chao, Scribner, Dixon & Malven, 1987).

Although the results of the experiments reported in this chapter show that exogenous NPY does not affect the reproductive axis of the sheep at the level of the hypothalamus, and those of Brooks *et al.* (1990) show no effect at the level of the pituitary, it is clear that exogenous NPY acts to stimulate the activity of the HPA axis in this species. We observed a clear stimulatory effect of i.c.v. NPY on this axis, assessed in terms of the secretion of cortisol, that was particularly effective in the luteal phase of the intact ewe and in the OVX/E_2 animal, but also to a lesser extent in the follicular phase. It seems likely, therefore, that the ability of NPY to affect this axis in the sheep is dependent upon the ovarian steroid environment, and in particular upon the presence of physiological levels of progesterone, for an optimal action. These results confirm and extend previous studies in the rat (Härfstrand *et al.* 1987) and dog (Inoue *et al.* 1989) that NPY has corticotrophin-releasing properties.

It seems most likely that the action of NPY in this regard is to stimulate the secretion of CRH within the hypothalamus (Haas & George, 1987; Tsaragarakis *et al.* 1989). The precise nature of the effect of NPY on the

neurone is not known, but the two types of cortisol secretion
ved after NPY administration in the luteal phase in the current
ment (Figure 8.8e–f) suggest two hypothetical possibilities. Firstly, it
be that NPY acts to cause the CRH neurone to depolarize completely
elease a large proportion of the contents of the nerve terminal
e 8.8e?). Secondly, NPY may act to modulate the electrical activity of
RH neurone in such a way, perhaps by depolarizing the membrane
tial only slightly, or by altering the threshold for release, that it
ies 'hyperexcitable' and fires more rapidly and with greater
tude than normal (Figure 8.8f?). These possibilities can only remain
lative until the neurotransmitter actions of NPY within the PVN are
igated electrophysiologically. However, it is of interest in this regard
i.c.v. NPY potentiates the effectiveness of central CRH as a
agogue for ACTH, acting at sub-threshold doses in synergism with
(Inoue *et al.* 1989). Whether NPY acts directly, or in synergy with

CRH, to release ACTH from the pituitary corticotrophs is unclear at present, although Inoue *et al.* (1989) found it to be only a weak ACTH-secretagogue when injected intravenously. These potential actions were not addressed in the present experiments, but could be investigated using, for instance, cultured pituitary cells.

In conclusion, the experiments described in this chapter demonstrate that, in contrast to data obtained primarily in the rat, LHRH/LH secretion is not under the regulatory control of NPY in the sheep. However, the results show quite clearly that NPY is involved in the multi-factorial regulation of ACTH and cortisol release in this species. In this chapter, the experiments reported addressed the effects of exogenous NPY. The next chapter describes experiments to investigate the role of endogenous NPY in the control of LH secretion, and in particular the role this neuropeptide may play with respect to the LH surge.

9. Role of endogenous neuropeptide Y in the secretion of LH during the oestrous cycle

9.1. Introduction

In the previous chapter, the actions of exogenous NPY on the hypothalamic-ovarian and -adrenal axes were reported. Whilst central NPY stimulated the HPA axis, the acute administration of NPY into the third ventricle was without effect on LH secretion at the doses tested. However, there is preliminary evidence in the rat that NPY may play a part in the initiation of the LH surge.

The concentration of NPY in the pituitary portal plasma of the rat is considerably higher than that in the peripheral plasma (McDonald, Koenig, Gibbs *et al.* 1987). Moreover, pituitary portal NPY levels change throughout the oestrous cycle in the rat (Sutton, Toyama, Otto & Plotsky, 1988), being particularly high on the afternoon of pro-oestrus. Passive systemic (Sutton *et al.* 1988) or central (Wehrenberg, Corder & Gaillard, 1989) immunization with antibodies to NPY inhibits the oestradiol- and progesterone-induced LH surge in the ovariectomized rat. In addition, it
en shown that hypothalamic ir-NPY increases during prepubertal
pment and that an increase in pituitary portal NPY levels precedes
st oestrous cycle of puberty in rats (Sutton, Mitsugi, Plotsky & Sarkar,
A recent study by this group demonstrated that immunization with
um against NPY suppresses the preovulatory surge of LH on the
on of first pro-oestrus and that a central, rather than peripheral,
of administration of antibody was more effective (Minami,
chy, Plotsky *et al.* 1990). These findings suggest that endogenous
ay play a physiologically important role in the initiation of the pre-
ory LH surge.
experiments reported in this chapter were designed to investigate
er endogenous NPY is important in the control of LH secretion in
eep oestrous cycle. We raised antibodies to NPY in sheep and
ined the effects of the peripheral and central (i.c.v.) administration
i-NPY antibodies on the timing and/or characteristics of the

oestradiol-induced surge in anoestrous ewes and of the pre-ovulatory LH surge in cycling ewes. In addition, we looked for evidence of the presence of NPY in the hypothalamus and attempted to measure the secretion of NPY throughout the cycle.

9.2 Experimental design and methods

The experiments described in this chapter involved four groups of intact ewes. Three groups of Scottish Blackface ewes were used (a) for measurement of NPY throughout the oestrous cycle; (b) to investigate the effect of peripheral administration of anti-NPY antibodies on the oestradiol-induced LH surge or (c) the pre-ovulatory LH surge. A group of Finn x Dorset ewes was also used in which the effect of i.c.v. administration of anti-NPY antibodies on the pre-ovulatory LH surge was measured.

In addition, four Scottish Blackface ewes were immunized against NPY to raise antibodies for the immunoneutralization studies.

9.2.1. Tissue extraction for measurement of neuropeptide Y content

Hypothalamic tissue was obtained from an intact control Scottish Blackface ewe (No. G253) in the early luteal phase. The animal was killed with an overdose of sodium pentobarbitone (Euthasate, Willows Francis, Crawley, West Sussex), the skull opened and the intact brain carefully removed leaving the pituitary gland *in situ*. The median eminence and
ıediobasal hypothalamus (MBH) were removed quickly from the
frozen rapidly on dry ice and stored at –70 °C. The MBH extended
the base of the brain to approximately 4–5 mm from the floor of the
ventricle dorsally, to the optic chiasm anteriorly, the mamillary
s posteriorly and to the optic tracts laterally. The median eminence
asily identified as a highly vascular ovoid structure, 2–3 mm in
ter, on the ventral surface of the hypothalamus.
ozen mediobasal hypothalamus or median eminence tissue was
l for 5 min in a mixture (1: 1) of acetic acid (1 M) and HCl (0.1 M)
l/g tissue). After cooling on ice, the tissues were homogenized
ron, Kinematica, Luzern, Switzerland), taking care to avoid cross-
nination, and an aliquot removed for protein determination using a
ercially available kit (BioRad, Munich, FRG) based on the method of
ord (1976). The homogenate was centrifuged at 3 000 rpm (2 110 *g*) for

60 min at 4 °C and the supernatant decanted and neutralized with 1 M $NaHCO_3$. The neutralized supernatant was then centrifuged at 3 000 rpm for 30 min and the resulting supernatant decanted and lyophilized under vacuum overnight. Freeze-dried tissue was reconstituted in assay buffer for determination of NPY content as below. I am grateful to Dr Fiona Gibson for help and advice with the tissue extractions, and for performing the protein assay.

9.2.2. Measurement of NPY during the oestrous cycle

During January 1989, the oestrous cycles of 10 Scottish Blackface ewes were synchronized prior to blood sampling in February. Initial cycle synchrony was by withdrawal of progestagen-impregnated intra-vaginal sponges 12 days after their insertion as described in the previous chapter (Section 8.2.4). All ewes displayed behavioural oestrus. On day 10 of the subsequent cycle, luteolysis was induced at 23.00 h with an i.m. injection of cloprostenol as previously. The animals were sampled every 10 min for 10 h from 09.00 h (10 h after cloprostenol) and subsequently every hour for a further 60 h until 07.00 h on the fourth day of sampling. Samples were collected on ice and the plasma separated and stored immediately at –20 °C until extracted for NPY assay.

Plasma samples (750 µl) were extracted with 3 ml HCl-ethanol (1.5 mM HCl in ethanol) in polystyrene tubes. Samples were vortexed for 30 sec and centrifuged at 3 200 rpm (2 400 *g*) for 15 min at 4 °C. The supernatant ecanted and dried down overnight in a water-cooled centrifugal rator (GyroVap, Howe, London) and reconstituted in 250 µl buffer ay of NPY as below. Extraction efficiency, as determined by recovery thetic NPY added to a freeze-thawed plasma pool was ~100% (range)%).

Radioimmunoassay of neuropeptide Y

nination of NPY content in tissue and in plasma samples was by a ed form of the methodology reported by McDonald and colleagues nald, Dees, Ahmed *et al.* 1987; McDonald, Koenig, Gibbs *et al.* 1987; no, Collins & McDonald, 1989). Briefly, the assay used a rabbit um raised against synthetic porcine NPY conjugated to BSA with aldehyde. This antiserum—NPY-3 from rabbit #3, April (4)/1985 –was a generous gift from Dr John K. McDonald, Emory University

School of Medicine, Atlanta, GA, USA, to whom we are grateful.

The assay was carried out in polystyrene tubes using 0.05 M phosphate buffer containing 0.3% BSA, 0.01 M EDTA and 0.2% sodium azide at pH 7.4. Human NPY (Cambridge Research Biochemicals, Harston, Cambridge) for use as standard was stored at –40 °C at a concentration of 200 ng/ml and diluted appropriately on the day of assay in RIA buffer. Mono-iodinated [^{125}I] synthetic porcine NPY, labelled with Bolton-Hunter reagent, was purchased from Amersham and stored in aliquots of 10 µl (1 µCi) at –20 °C until required for assay.

100 µl of standard (0.1–25 ng/ml) or extracted tissue/plasma sample (see above) was incubated with 100 µl anti-NPY antiserum (1: 9 000) and 100 µl [^{125}I]-pNPY (~7 000 cpm/100 µl) for 24 h at 4 °C. The bound fraction was then separated by incubating overnight at 4 °C with 100 µl donkey anti-rabbit serum (1: 16) and 100 µl normal rabbit serum (1: 800). 1 ml 0.9% saline/0.02% Triton X-100 was then added and the tubes centrifuged. The supernatant was decanted and the remaining pellet counted as described previously (Chapter 3). Binding in the presence of unlabelled NPY was typically 25–30% and the non-specific binding was 2%. The sensitivity of the assay was 20 pg/tube and, after the concentration step in the plasma extraction, the detection limit was 67 pg/ml. (Representative standard curve shown in Figure 9.3).

9.2.4. Generation of anti-NPY antibodies

e primary immunization and the first boost, NPY was conjugated to γ-globulin (rγG). However, rγG is *not* to be recommended as a ı. The in-house ovine gonadotrophin assays (and many others) use ːy antisera raised in rabbits, and so the anti-rabbit antibodies raised ɔy-product' of conjugation to rγG will cause the assay to produce us high concentrations when none exist. The anti-rabbit antibodies ɔre had to be removed from the anti-NPY antiserum before use (see). Other haptens such as bovine or porcine thyroglobulin or keyhole : haemocyanin would avoid this problem and indeed porcine ;lobulin (pTG) was used for subsequent boosts. The method ɔed below was that for conjugation to pTG, but the same principle llowed for rγG.

man NPY was conjugated to porcine thyroglobulin (Sigma) in a ratio of 5: 1 with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide ːhloride (carbodiimide; Sigma). 400 µg hNPY and 12.5 mg pTG were

dissolved in 1 ml sterile saline (pH 5.5–6.0). 30 mg fresh carbodiimide was dissolved in 400 µl distilled water and added to the proteins. The mixture was then agitated at room temperature for 4 h, before dialysis against 5 l 0.9% saline for 24 h, replenishing the saline at least three times. For immunization and subsequent boosts, the NPY conjugate was made up to 4 ml with saline and and emulsified with 6 ml Freund's complete adjuvant (GIBCO Laboratories, Grand Island, NY, USA). The emulsification and injections were made using a 5.0-ml Eppendorf Multipipette combitip (Eppendorf-Netheler-Hinz GmbH, Hamburg, FRG) trimmed to a fine aperture for the emulsification and cut down to accept a 19-gauge needle for the injections.

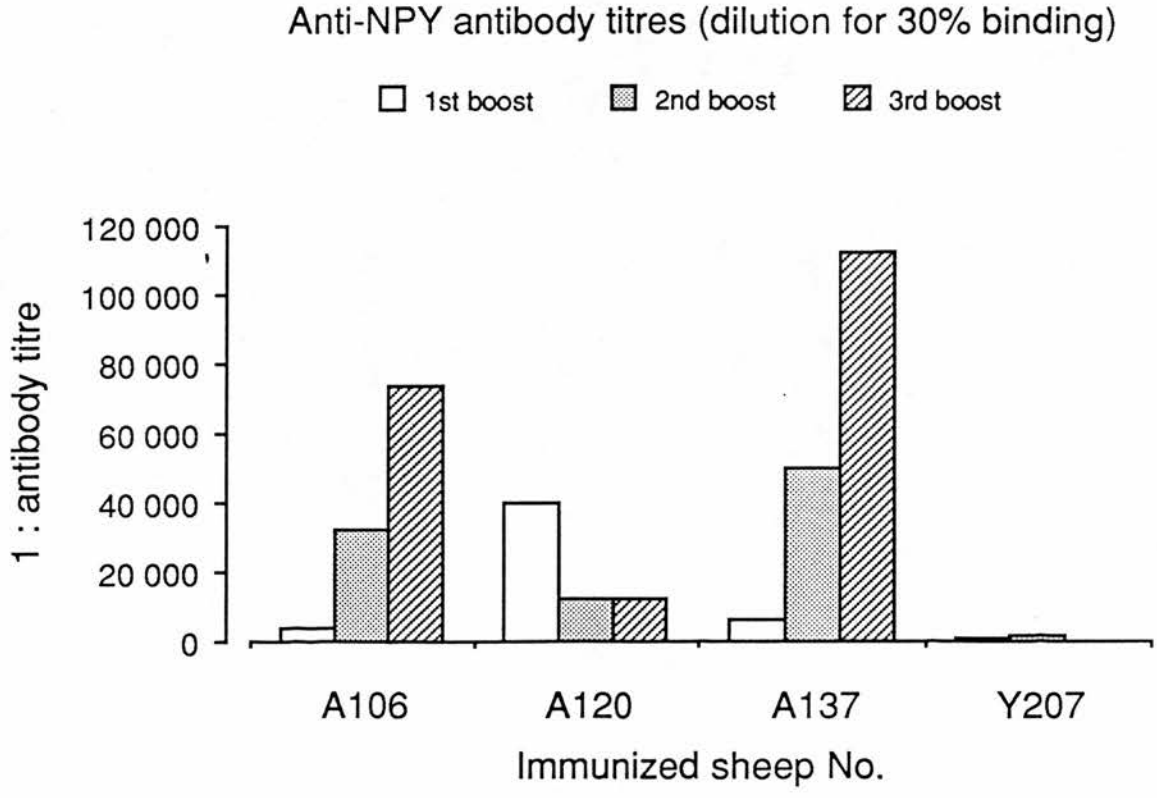
Four Scottish Blackface ewes were injected intradermally in the axillary skin at multiple sites with 100 µg NPY conjugate emulsified with Freund's (total volume 2.5 ml). The sheep were boosted at 10, 15, 20 and 30 weeks after the initial immunization, again with 100 µg NPY conjugate emulsified with complete Freund's. 12 days after each boost, 400 ml blood was collected from each sheep and the plasma stored at –20 °C. The antibody titres were determined by incubating dilutions of these samples with ^{125}I-NPY. Bound tracer was separated from free with polyethylene glycol (PEG). 1 mg rγG in 100 µl buffer and 1 ml 20% PEG/0.02% Tween in buffer was added to each tube and, after vortexing thoroughly, the tubes were centrifuged at 3 000 rpm for 30 min at 4 °C. The supernatant was aspirated and the remaining white pellet counted. As shown in Figure 9.1,

he third boost (07.06.89 bleed), one sheep (No. A137) had a titre of 000 (defined as the final dilution of antiserum required to bind 30% -labelled NPY trace). The titre of this animal after the fourth boost 89 bleed) was similar, and antibodies from both these bleeds were n the immunoneutralization studies described in this chapter.

Removal of anti-rγG antibodies from anti-NPY antiserum

to use, the antiserum was cleaned of anti-rabbit antibodies (see) by passing it through an agarose gel column containing rγG (Sigma) ed to the gel by the following method. 15 g CnBr-activated Sepharose (Pharmacia AB, Uppsala, Sweden) was swollen for 15 min in 1 mM nd then washed and reswollen with 1 mM HCl on a sintered glass The gel was then washed with 75 ml coupling buffer (0.1 M CO_3/0.5 M NaCl; pH 8.3) and immediately transferred to a solution of g rabbit γ-globulin in coupling buffer. The protein solution was

Figure 9.1. Titres of anti-NPY antibodies measured in four immunized sheep after the first three boosts with conjugated NPY and Freund's complete adjuvant (see text). After the third boost, sheep A137 had a titre of 1: 112 000.


Anti-NPY antibody titres (dilution for 30% binding)
1st boost
2nd boost
3rd boost
1 : antibody titre
120 000
100 000
80 000
60 000
40 000
20 000
0
A106
A120
A137
Y207
Immunized sheep No.

mixed gently with the gel suspension for 2 h at room temperature and the remaining active groups blocked with 0.2 M glycine (pH 8.0). Excess adsorbed protein was washed away by washing alternately 4–5 times with high-pH coupling buffer and low-pH acetate buffer (0.1 M sodium acetate/0.5 M NaCl; pH 4.0). The coupled gel was stored in 0.075 M phosphate buffer until required.

To remove the anti-rγG antibodies from the anti-NPY antiserum, it was passed through the column very slowly 2–3 times. The anti-rgG antibodies were eluted from the column with 0.1 M glycine/HCl (0.1 M glycine adjusted to pH 2.5 with 0.2 M HCl) and, after washing the column with phosphate buffer, the antiserum was passed through again. This treatment was successful in removing rγG-binding activity from the antiserum. Titres of anti-NPY antibodies were unaffected.

9.2.6. Systemic immunization against NPY during the oestradiol-induced LH surge in anoestrous Scottish Blackface ewes

During June 1989, twelve Scottish Blackface ewes were housed for experiment in approved restraint crates in the University of Edinburgh Marshall Building. Two days before, and on the day before experiment, the animals received either 10 ml anti-NPY plasma (A137; 07.06.89 bleed) (n = 6) or 10 ml non-immune plasma (n = 6) at 12.00 h by slow intravenous injection. On the day prior to experiment, a jugular venous cannula was inserted into each ewe and kept patent with heparinized

. At 00.00 h on the day of experiment, each animal received an i.m. on of 50 μg oestradiol benzoate (E_2B; Sigma) and a blood sample (2.5 as taken. 6 h later, at 06.00 h, the sheep received a third i.v. injection ibodies (or non-immune plasma) as previously and hourly blood ing (2.5 ml) for 24 h was commenced. Plasma was separated and at –20 °C until assayed for LH. The time after E_2B to the onset of the rge was defined as in Section 8.2.4.

Systemic immunization against NPY during the pre-ovulatory LH in cycling Scottish Blackface ewes

g October–November 1989, the oestrous cycles of 20 Scottish ace ewes (47–75 kg) were synchronized prior to experimentation in nber. Initial cycle synchrony was by withdrawal of progestagen-gnated intra-vaginal sponges 12 days after their insertion as

described previously (Section 8.2.4). All ewes displayed behavioural oestrus. On day 10 of the subsequent cycle, a jugular venous cannula was inserted into each ewe and kept patent with heparinized saline. The animals were placed in approved restraint crates for experiment and luteolysis was induced at 23.00 h with an i.m. injection of cloprostenol. 10 h after cloprostenol, i.e. at 09.00 h, the animals received either 8 ml anti-NPY antiserum (A137; 21.08.89 bleed) (n = 10) or non-immune plasma (n = 10) by slow intravenous injection. These injections were repeated at 09.00 h on the following two days. Blood samples (2 ml) were taken every 10 min for 10 h from 09.00 h (10 h after cloprostenol) and subsequently every hour for a further 60 h until 07.00 h on the fourth day of sampling. Plasma was separated and stored at –20 °C until assayed for LH. The time to the onset of the pre-ovulatory LH surge was defined as in Section 8.2.4.

9.2.8. Central (i.c.v.) immunization against NPY during the pre-ovulatory LH surge in cycling Finn x Dorset ewes

Four of the Finn x Dorset ewes described in Section 8.2.4 were used for this part of the study during January–February 1990. After the experiments on these animals reported in the previous chapter, all ewes showed behavioural oestrus. At 21.00 h on day 9 of the following cycle, each animal received an injection of 50 µl non-immune plasma into the third cerebral ventricle. The next day (day 10), a jugular venous cannula for blood sampling was inserted into each sheep and each received a further

injection of control plasma. Luteolysis was induced with i.m.
stenol at 23.00 h. At 09.00 and 21.00 h on each of the three following
the animals received an i.c.v. injection of 50 µl non-immune
a. Starting 10 h after cloprostenol (09.00 h), blood samples (0.8–1.0
ere taken every 10 min for 10 h and subsequently every hour for the
0 h. All animals showed behavioural oestrus. Due to the small
e volume collected, blood was centrifuged in small heparinized
without beads and the plasma decanted by pipette.

e animals were allowed two complete oestrous cycles for recovery,
; which they did not receive any treatment. They were then given
v. injection of 50 µl anti-NPY antiserum (A137; 07.06.89 bleed) at
n on day 9 of the subsequent cycle. The i.c.v. injection of anti-NPY
um was repeated at 21.00 h the next day and at 09.00 and 21.00 h on
lowing three days, as above for control plasma. On the afternoon of
), a jugular venous cannula was inserted into each animal and

luteolysis induced at 23.00 h with i.m. cloprostenol. Blood samples (0.8–1.0 ml) were taken every 10 min for 10 h from 09.00 h (10 h after cloprostenol) and subsequently every hour for the next 60 h. Figure 9.2 shows the times of injection of antibody in relation to the stage of the oestrous cycle as illustrated by progesterone levels measured in plasma.

9.3. Results

9.3.1. Hypothalamic NPY content

Serial dilutions of extracts of mediobasal hypothalamus and median eminence exhibited close parallelism with synthetic human NPY standards in the radioimmunoassay (see Figure 9.3), demonstrating NPY-immunoreactivity in the hypothalamus of the sheep.

The content of NPY in the MBH was 489 ng/g tissue and, in the ME, 336 ng/g tissue. As the MBH and ME contained 63.7 and 23.4 mg protein/g tissue respectively, these values correspond to concentrations of NPY of 3.68 μg/g protein in the MBH and 14.3 μg/g protein in the ME, or 0.86 and 3.36 pmol NPY/mg protein respectively.

9.3.2. Plasma concentrations of NPY during the oestrous cycle

After several abortive attempts to extract and measure NPY in plasma samples taken prior to and during the expected time of the pre-ovulatory
rge, we obtained the profile shown in Figure 9.4. With the caveat
is is only one profile from a single animal, this appears to show that
a concentrations of NPY increase around the time that the pre-
ory surge of LH might be expected to occur and also that NPY
on earlier in the cycle may be pulsatile.
fortunately, it is not known when the LH surge occurred in this
ılar ewe, as the whole sample was required for extraction due to the
ow concentration of NPY in the peripheral plasma. In addition, this
eant that the NPY assay could not be performed in duplicate. It is
ore impossible to draw any conclusions from this part of the study.

Effects of systemic passive immunization against NPY

enous administration of anti-NPY antibodies in anoestrous Scottish
ıce ewes had no effect on the E_2B-induced LH surge. The time from
istration of E_2B to the onset of the LH surge was not altered (14.2 ±

Figure 9.2. Progesterone concentrations in intact cycling ewes, showing the timing of i.c.v. administration (see text) of control plasma (open bar) or anti-NPY antibodies (shaded bar) relative to the oestrous cycle(s). ● = mean plasma progesterone levels in Finn x Dorset ewes during the experiment ± s.e.m. (n = 4). Injections of cloprostenol shown by open arrows. See also legend to Figure 8.1.

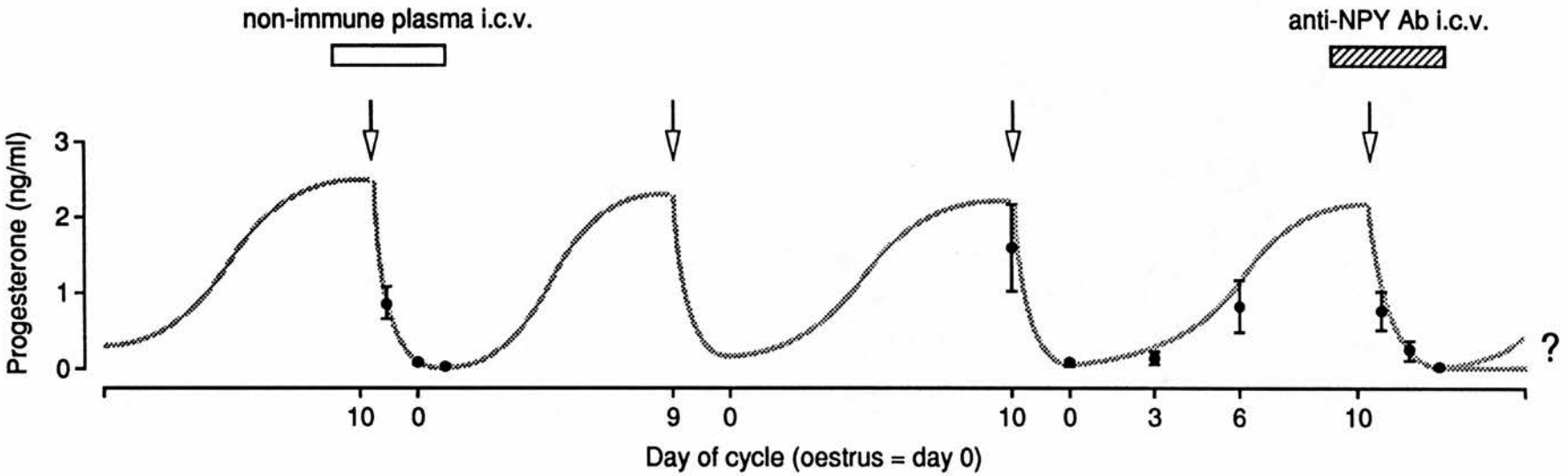

non-immune plasma i.c.v.
anti-NPY Ab i.c.v.
Progesterone (ng/ml)
3
2
1
0
10
0
9
0
10
0
3
6
10
?
Day of cycle (oestrus = day 0)

Figure 9.3. Content of immunoreactive NPY in (□) medio-basal hypothalamus and (■) median eminence. Dilutions of tissue extracts show parallelism with hNPY standard curve (●). Tissue obtained from a control ewe during the early luteal phase.

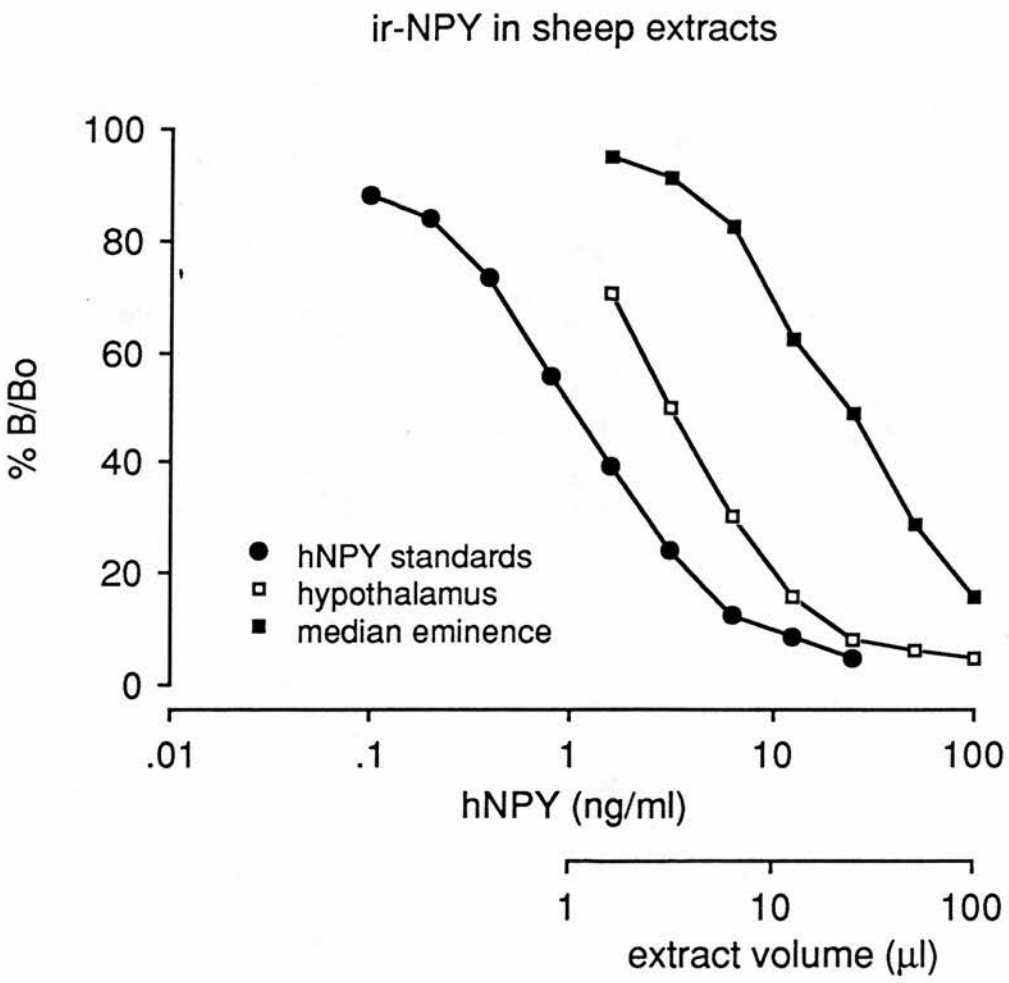
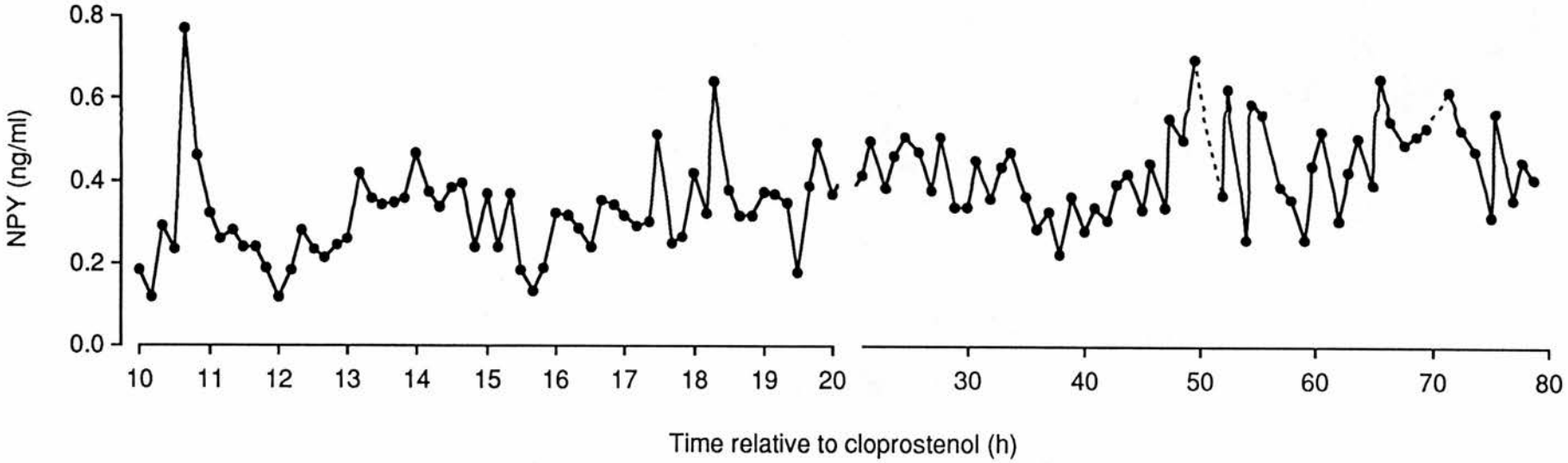

ir-NPY in sheep extracts
100
80
60
40
20
0
% B/Bo
hNPY standards
hypothalamus
median eminence
.01
.1
1
10
100
hNPY (ng/ml)
1
10
100
extract volume (µl)

Figure 9.4. Measurement of NPY in extracted peripheral plasma before and during the expected time of the pre-ovulatory LH surge. Note split and change of scale of x-axis after t = 20 h. Time measured from induction of luteolysis with cloprostenol (see text).


0.8
0.6
0.4
0.2
0.0
NPY (ng/ml)
10
11
12
13
14
15
16
17
18
19
20
30
40
50
60
70
80
Time relative to cloprostenol (h)

0.44 h in antibody-treated animals, compared with 15.2 ± 1.11 h in ewes treated with non-immune plasma; n = 6 in each group). Other characteristics of the surge, such as the peak height (Ab-treated, 144.1 ± 26.1 ng/ml vs. control, 106.2 ± 17.3 ng/ml), were also not altered (Figure 9.5a).

Passive intravenous immunization against NPY during the pre-ovulatory LH surge in Scottish Blackface ewes, though, did reduce the time to the onset of the surge in cycling animals (Ab-treated, 48.8 ± 2.69 h vs. control, 58.5 ± 2.24 h; n = 10 in each group; $p < 0.05$, Neuman-Keuls test). Other characteristics of the surge, however, including peak height (Ab-treated, 95.8 ± 13.4 ng/ml vs. control, 81.1 ± 11.0 ng/ml), were unaltered (Figure 9.5b).

Although there was no difference in the mean weights of the two groups of animals (Ab-treated, 56.7 ± 2.1 kg vs. control, 61.2 ± 2.0 kg), it seems likely that the reduction in time to onset of the surge seen with immunization against NPY may be due to a confounding effect of the nutritional state of the animal (see Section 9.4 below).

9.3.4. Effects of central (i.c.v.) immunization against NPY

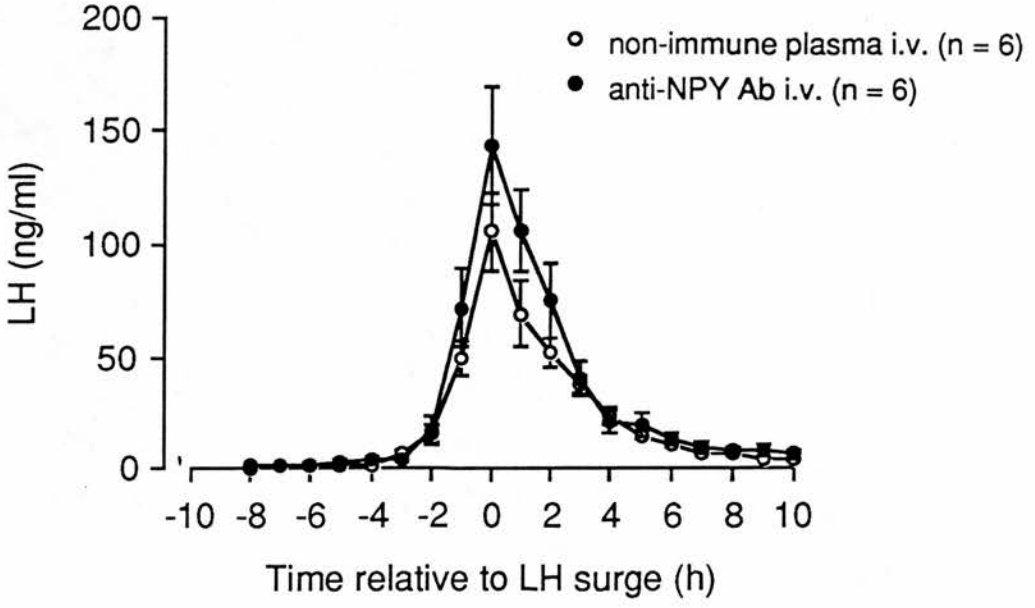
In contrast to the effects of peripheral NPY-Ab treatment, i.c.v. administration of anti-NPY antibodies prior to and during the expected time of the pre-ovulatory surge in Finn x Dorset ewes delayed or apparently abolished the LH surge in all three ewes for which we were able to obtain both control and treated data. Figure 9.6 shows the LH profiles of

three ewes treated with anti-NPY antibodies i.c.v. and also the
es of the same animals treated with non-immune plasma i.c.v. for
arison. Interestingly, three out of the four ewes did not show
ioural oestrus during the NPY-Ab treatment, and the fourth (No.
3) did not allow the ram's attempted mounts to succeed.

ie to the nature of the data, and as we were only able to obtain a
ete set from three animals, it was not possible to perform a statistical
iis. We were, however, able to obtain a complete data set from the
0 h of sampling (i.e., from 10–20 h after cloprostenol) and found
to be no effect of NPY immunization on mean basal cortisol
ntration over this time period (Ab-treated, 24.6 ± 3.68 ng/ml vs.
l, 28.1 ± 3.64 ng/ml; n = 4 in each group).

Figure 9.5. Passive systemic immunization with anti-NPY antiserum had no effect on the characteristics of (a) the oestradiol-induced surge of LH in anoestrous Scottish Blackface ewes or (b) the pre-ovulatory LH surge in cycling Scottish Blackface ewes. The means (± s.e.m.) of the LH profiles relative to the peak concentration of LH are shown for each treatment. (a) effect of i.v. administration of (○) non-immune plasma (n = 6) and of (●) anti-NPY antibodies (n = 6) on the oestradiol-induced LH surge in anoestrus. (b) effect of i.v. treatment with (○) control plasma (n = 10) and (●) anti-NPY antibodies (n = 10) on the pre-ovulatory surge in cycling ewes. LH concentration expressed in terms of NIH-LH-S23.

(a) Oestradiol-induced LH surge in anoestrous ewes



(b) Pre-ovulatory LH surge in cycling ewes

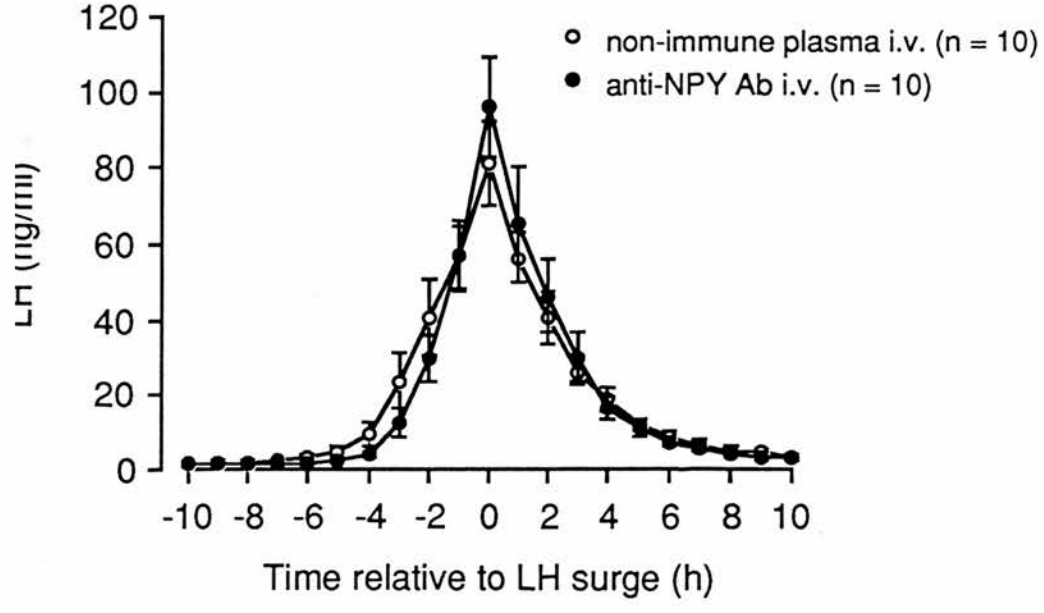
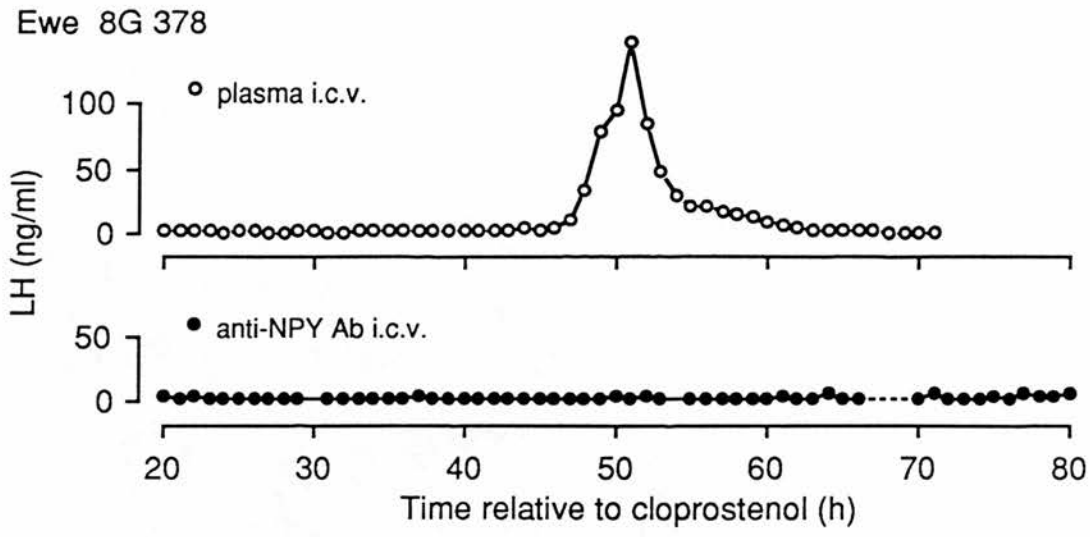
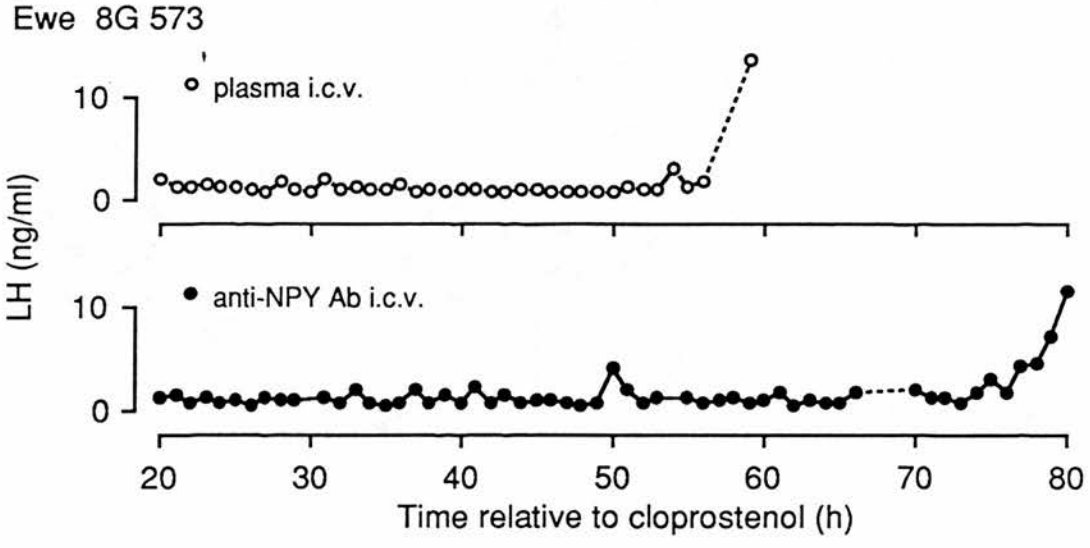
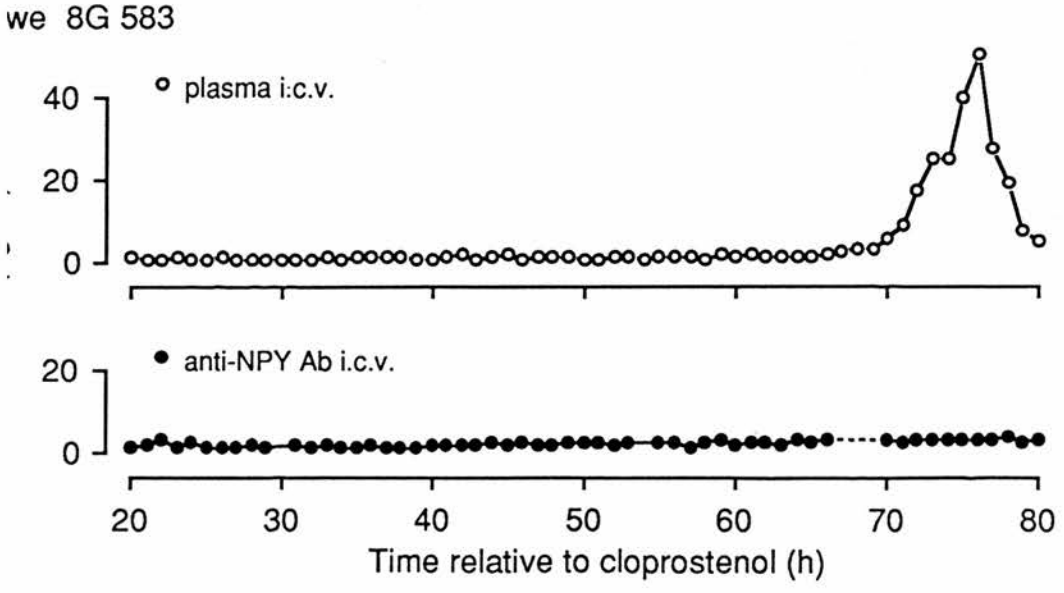


Figure 9.6. Effect of i.c.v. administration of anti-NPY antibodies (see text) on the timing of the pre-ovulatory LH surge in three cycling Finn x Dorset ewes. The effects of (○) control treatment with non-immune plasma and (●) administration of anti-NPY antiserum are shown. Central administration of anti-NPY antibodies delayed the LH surge in all animals. LH concentrations expressed in terms of NIH-LH-S23.


Ewe 8G 378
plasma i.c.v.
100
50
0
LH (ng/ml)
anti-NPY Ab i.c.v.
50
0
20
30
40
50
60
70
80
Time relative to cloprostenol (h)


Ewe 8G 573
plasma i.c.v.
10
0
LH (ng/ml)
anti-NPY Ab i.c.v.
10
0
20
30
40
50
60
70
80
Time relative to cloprostenol (h)


we 8G 583
plasma i.c.v.
40
20
0
anti-NPY Ab i.c.v.
20
0
20
30
40
50
60
70
80
Time relative to cloprostenol (h)

9.4. Discussion

We have clearly shown that ovine hypothalamic and median eminence tissues contain high concentrations of neuropeptide Y. While in the present study tissue from only one animal was used, the content of NPY in these tissues was within an order of magnitude of the concentrations shown in the rat brain (Chronwall *et al.* 1985). These authors applied a rigorous and detailed quantitative analysis to micro-dissected brain regions in a large group of rats. The present study did not set out to be rigorous, and nor could the brain areas be described as micro-dissected. However, within the aim of the study, we have demonstrated the presence of ir-NPY in these hypothalamic areas and that this immunoreactivity dilutes in parallel with human NPY standards. These results are suggestive that hypothalamic NPY could have a role in brain function in the sheep.

Our attempts at measuring NPY in peripheral plasma unfortunately did not reveal any conclusive data. To get the extremely low peripheral concentrations of NPY to read on the standard curve of the RIA, large volumes had to extracted and concentrated up. This had the disadvantage that samples could not be assayed in duplicate and nor was there enough sample to assay for LH. This is unsatisfactory from the point of view of relating NPY levels to the stage of the oestrous cycle. From the one profile

ve were able to obtain, it appears that NPY concentrations may
ly rise around the expected time of the surge and that NPY secretion
have a pulsatile component. These conclusions are entirely
ative until a better method of assessing endogenous NPY secretion
found.

eral ways of overcoming these problems could be suggested. In the
PY levels have generally been reported in pooled plasma samples
Sutton *et al.* 1988; McDonald *et al.* 1987) which gets round the
m with, however, considerable loss of resolution. An immuno-
netric assay (IRMA) would better overcome the problems of low
trations, as in general IRMAs are much more sensitive. An NPY
has been reported by Corder & Lowry (1985) and, if combined with
clonal antibody technology, such an assay would have distinct
tages over RIA.

e approach that would reveal the greatest amount of physiologically

interesting information regarding the secretion of NPY would be to measure NPY in serial samples of portal blood using an IRMA. Techniques for the acute collection of portal plasma in the sheep have been developed by Clarke & Cummins (1982) and further refined by Caraty & Locatelli (1988). These would be ideally suited to investigation of NPY secretion from the hypothalamus without interference from NPY from peripheral sources such as the ovary, adrenals, or the blood vessel smooth musculature.

A much less traumatic or invasive technique with significant advantages over the surgical trans-nasal/-sphenoidal approaches has been developed for use in the horse (Irvine & Alexander, 1987). This takes advantage of the unique venous drainage pathway of the pituitary gland in this species and allows for collection of essentially portal blood samples of large volume (up to 4 ml) at frequent intervals (e.g. every 2 min) for up to several days. There are, however, some disadvantages to this model. Firstly, the blood collected is venous *effluent*—i.e., *after* it has passed through the pituitary gland—and presumably a proportion of the hypothalamic hormones will be internalized by the cells of the anterior pituitary. Secondly, due to the unique anatomy of the venous pathway used, it is only possible in the horse, which is a species not widely used as a neuroendocrine model. Nevertheless, this would be an ideal model in which to investigate the hypothalamic secretion of NPY through the oestrous cycle.

In order to investigate the possible role of endogenous NPY in the l of LH secretion at the pituitary and hypothalamic levels, we raised dies to NPY and administered them both peripherally and centrally g either the E_2B-induced LH surge in anoestrous ewes (peripheral or the pre-ovulatory surge in cycling ewes. Systemic treatment with PY antibodies had no effect on the induction of a surge of LH by i.m. This is in contrast to data obtained in the ovariectomized rat (Sutton 1988), where i.v. treatment with anti-NPY antibody inhibited the diol- and progesterone-induced surge of LH. However, it is not sing as NPY has no effect at the level of the pituitary gland in the (Brooks *et al.* 1990). It appears also that the peripheral admin-on of antibody in this pharmacological model does not affect the le modulation by NPY of LHRH release from circumventricular s outside the blood-brain barrier, such as the median eminence and VLT.

Systemic immunization against NPY during the pre-ovulatory LH surge appeared to reduce the time to surge onset without, however, altering the characteristics of the surge. It seems that this may be due to a confounding effect of the nutritional state of the animals. Whilst the weights of the two groups were not different, the antibody-treated group was composed, in the main, from animals recently brought in from pasture, whereas the majority of the control animals had been housed indoors in the sheep facility for several months on a diet of hay, supplemented with concentrated feed. A lower body condition (degree of fatness) in hill and upland sheep, such as the Scottish Blackface, has been shown to be associated with reduced numbers of large follicles, with a lower proportion of oestrogenic, potentially ovulatory, follicles among this reduced population (McNeilly, Jonassen & Rhind, 1987). If the body condition of the outdoor sheep was significantly higher for the same weight, with a consequentially increased follicular development, then this provides the only plausible explanation for the difference in the time to onset of the LH surge.

In contrast to the effects of systemic (peripheral) immunization against NPY, i.c.v. administration of anti-NPY antibodies resulted in a delay in the onset of the LH surge in all animals for which we were able to obtain control and treated data. This is in agreement with the findings of Minami *et al.* (1990) with regard to the onset of the first LH surge of puberty in the rat. These authors observed two populations of effect of NPY-Ab on the surge; in some animals the surge was abolished the observation period, whereas in others it was only delayed. As ion occurred in all the treated rats, except for one, on the next ng, they concluded that a surge of LH had perhaps occurred after the the sampling period. It is possible that this was the case in our Ab-treated sheep. However, the absence of behavioural oestrus and ie presence of apparently anoestrous ovaries in one of the ewes on ctomy shortly after the completion of the experiment (Dr Alan S. lly, personal communication) suggests either that an LH surge did cur, or if it did, it was not sufficient to produce ovulation. The breed ep used for the central experiment—the Finn x Dorset—does not y enter anoestrus until later in the year, as it has been specifically or, amongst other attributes, a longer breeding season. The delay in of the pre-ovulatory LH surge, or its abolition, caused by i.c.v. ization against NPY suggests that endogenous NPY plays a part in

the modulation of the timing of the LH surge in the sheep, and that this occurs at the level of the hypothalamus. It may be that NPY acts to synchronize or optimize the firing of LHRH neurones in such a way that this contributes to the creation of the LH surge. Alternatively, NPY neurones could act in an interneurone capacity to regulate the response of the LHRH neurones to circulating levels of oestradiol; i.e., they may influence the 'set-point' of oestradiol positive feedback. Either or both of these explanations could account for our findings.

Although the experiments of the previous chapter and those of Inoue *et al.* (1989) in the dog show that NPY is involved in the multi-factorial regulation of ACTH release, cortisol levels were found to be unchanged during the central immunization against NPY. This suggests that NPY is not important for basal cortisol release. It would be of interest, however, to determine the effects of anti-NPY immunization on stimulated cortisol release as, for instance, in hypoglycaemic stress or in response to an audio-visual stimulus such as a barking dog. This would address the physiological significance of endogenous NPY in the activation of the HPA axis.

In conclusion, we have shown that endogenous NPY is involved in the modulation of the timing of the LH surge in the sheep, and that in contrast to the role of NPY in this regard in the rat, this only occurs at the level of the hypothalamus. It is not possible from the current experiments to determine whether this role of NPY is an *obligatory* part of the surge-

ting mechanism. However, the effects of central NPY immun-

in the rat suggest that, at least in this species, NPY plays a

tory role in the generation of the LHRH surge. It may be, therefore,

e function of NPY in the regulation of reproduction in the sheep is

vide a means whereby the efficient generation of a pre-ovulatory

/LH surge is ensured.

10. General discussion

The primary control of reproduction is through the action of the hypothalamic releasing hormone LHRH, released in a pulsatile fashion into the pituitary portal system. The *generation* of LHRH pulses is clearly an intrinsic property of the LHRH neuronal system as LHRH pulses continue at a physiological frequency if the human MBH is placed in an in-vitro perifusion system (Rasmussen, Gambacciani & Swartz *et al.* 1989). However, afferent pathways that project to LHRH cells are important for the *modulation* of LHRH pulses. The experiments reported in this thesis have shown that other neuropeptides have the ability to regulate this primary drive and that the intra-hypothalamic interactions between neuropeptides involved in the control of the reproductive axis are diverse and complex.

The findings of Chapter 4 (see also Naylor, Porter & Lincoln, 1989) indicate that an inhibitory autoregulatory mechanism exists in the control of LHRH within the hypothalamus, and confirm and extend the results of experiments in the rat. The functional significance may be to provide a mechanism to switch off each pulse of LHRH. Experiments by Stephen Kuffler and colleagues have identified LHRH as a peptide neuro-

ıitter in the sympathetic ganglion cell of the bullfrog. Stimulation afferent nerves to this preparation results in two excitatory post-ic potentials, a fast synaptic potential mediated by acetylcholine and much slower potential that lasts for several minutes (see Kuffler, lls & Martin, 1984). LHRH-ir was found to increase after electrical ation of the pre-ganglionic fibres and exogenously applied LHRH ked the effects of nerve stimulation (Jan, Jan & Kuffler, 1979; 1980; Jan, 1982). Moreover, application of an LHRH antagonist blocked he late slow potential and the LHRH-induced potential (Jan *et al.* Jan & Jan, 1982). Furthermore, Kuffler & Sejnowski (1983) ıstrated that the amplitude and duration of conductance changes ced by the neurally released transmitter and by LHRH on the naptic cell were indistinguishable. These experiments provide clear ıce that LHRH acts as a neurotransmitter in this preparation, ting late and long lasting excitatory actions on the postsynaptic cell

membrane. If the effect of LHRH upon LHRH neurones is characterized by such a slow onset, long-lasting action on ion channels, but to *hyper*polarize the cell membrane, then—as suggested by Dyer & Robinson (1989)—this could provide a mechanism for timing the release of LHRH pulses. The experiments reported here are unable to answer this specific question. Detailed electrophysiological studies of hypothalamic POA slices, or of cultured neurones are now required to determine the autoregulatory effects of LHRH at the neuronal level.

In a very recent paper, Caraty, Locatelli, Delaleu *et al.* (1990) have suggested that an autoregulatory mechanism in the control of LHRH secretion does not exist. Their conclusions were based on the measurement of endogenous LHRH in the portal blood of short-term castrated rams: during the peripheral (intramuscular) administration of an LHRH agonist and an LHRH antagonist, LHRH secretion continued, whilst LH secretion was interrupted. However, it is erroneous to infer from these particular findings that an intra-hypothalamic 'ultra-short' loop feedback mechanism does not exist for LHRH. As peptides do not cross the blood-brain barrier to any great extent, due to the non-fenestrated nature and the tight endothelial junctions of the brain capillaries (see Dunn & Berridge, 1987), this approach only addresses the effects of the LHRH analogues on the pituitary gland. Evidence *for* LHRH autoregulation within the hypothalamus is much more convincing (see Chapter 4).

Caraty *et al.* (1990) also raised the question of *dose*, implying that the
ity of LHRH required to produce an inhibitory effect when injected
lly was very high when compared with levels of LHRH in the CSF.
ppropriate dose of a neuropeptide is indeed problematic, yet it is
known what the physiologically effective concentration of a
peptide is, nor is it known what concentration of the administered
le reaches the active site(s). Interestingly, at the neuromuscular
on, it has been estimated that the concentration of acetylcholine
one released quantum is 0.3 mM (Kuffler & Yoshikami, 1975); which
above the K_d for the muscle acetylcholine receptor. Even if the K_d of
ceptor were taken as an estimate, then the effective concentration
l correspond to 10^{-12} to 10^{-9} M, depending on the affinity of the
or, or a dose of 1 pmol to 1 nmol distributed over the entire rat brain
& Berridge, 1987). For a decapeptide such as LHRH, this would
e a dose of between 1 ng and 1 μg in the rat. The doses used in the
t studies are thus well within the active range.

The temporal characteristics of the inhibition of LH secretion induced by central administration of LHRH suggested the possible involvement of another neural or endocrine system. Although the potential roles of the endogenous opioid peptides and the hypothalamo-pituitary-adrenal axis were investigated, the work reported here is not conclusive regarding the direct or indirect nature of LHRH autoregulation. The results presented in Chapter 5 indicate that the EOPs do not mediate the inhibition, and those in Chapter 6 suggest that cortisol, if it is implicated, is unlikely to be the sole factor involved in the suppression. The stimulatory effects of CRH on LHRH, and therefore on LH secretion, reported in Chapter 7 indicate that CRH is unlikely to suppress LHRH secretion centrally in the sheep. However, it is possible that CRH and/or AVP may be involved in some other pathway to inhibit LHRH secretion, not mimicked by i.c.v. administration of CRH. One way to determine this *in vivo* could be to administer anti-CRH or anti-AVP antibodies, or antagonists of either hormone centrally, prior to the ventricular injection of LHRH. In addition, the potential role of ACTH could be investigated further *in vivo*, by immunization against this hormone.

Another neuroendocrine mechanism not investigated in the current studies, but of interest as a potential mediator of the LHRH-induced suppression of LHRH/LH secretion, is the endogenous GABAergic system. Many of the LHRH neurones in the anterior hypothalamus have been shown to receive a GABAergic input (Leranth, MacLusky, Sakamoto *et al.* 1985). There is also much evidence in the rat for an action of GABA on secretion which is dependent both on the site of action within the ıalamus (Nikolarakis, Loeffler, Almeida & Herz, 1988) and on the environment (Jarry, Perschl & Wuttke, 1988). Moreover, GABA a reduction in plasma LH levels when injected into the third le of the rat (Fuchs, Mansky, Stock *et al.* 1984). Therefore, this is a that warrants further investigation *in vivo* and *in vitro*.

results of the experiments described in Chapter 7 indicate that in e, the central application of CRH causes an increase in the pulsatile ɔn of LHRH. As discussed in that chapter, this is in contrast to the ɔtained in the rat. However, this does not discount the theory that ıay also act to *inhibit* LHRH secretion in the sheep under some stances. The dominant pathway mimicked by the central admin-ɔn of CRH appears to be a neural circuit(s) involved in the ıtion of neuroendocrine responses to stress. The physiological

significance may be that such a mechanism underlies the LH responses to shipping/handling stress. An interesting area of further study in this respect would be to investigate whether the activation of such CRH pathways is involved in the so-called 'ram effect'. During anoestrus, if ewes previously isolated from the presence of rams are exposed to a ram, this results in the stimulation of the frequency of LH pulses (e.g., see Martin & Scaramuzzi, 1983; Martin, Scaramuzzi & Lindsay, 1983). This seems to be mediated via pheromones, but could also provide a physiological role for CRH to stimulate LHRH/LH secretion.

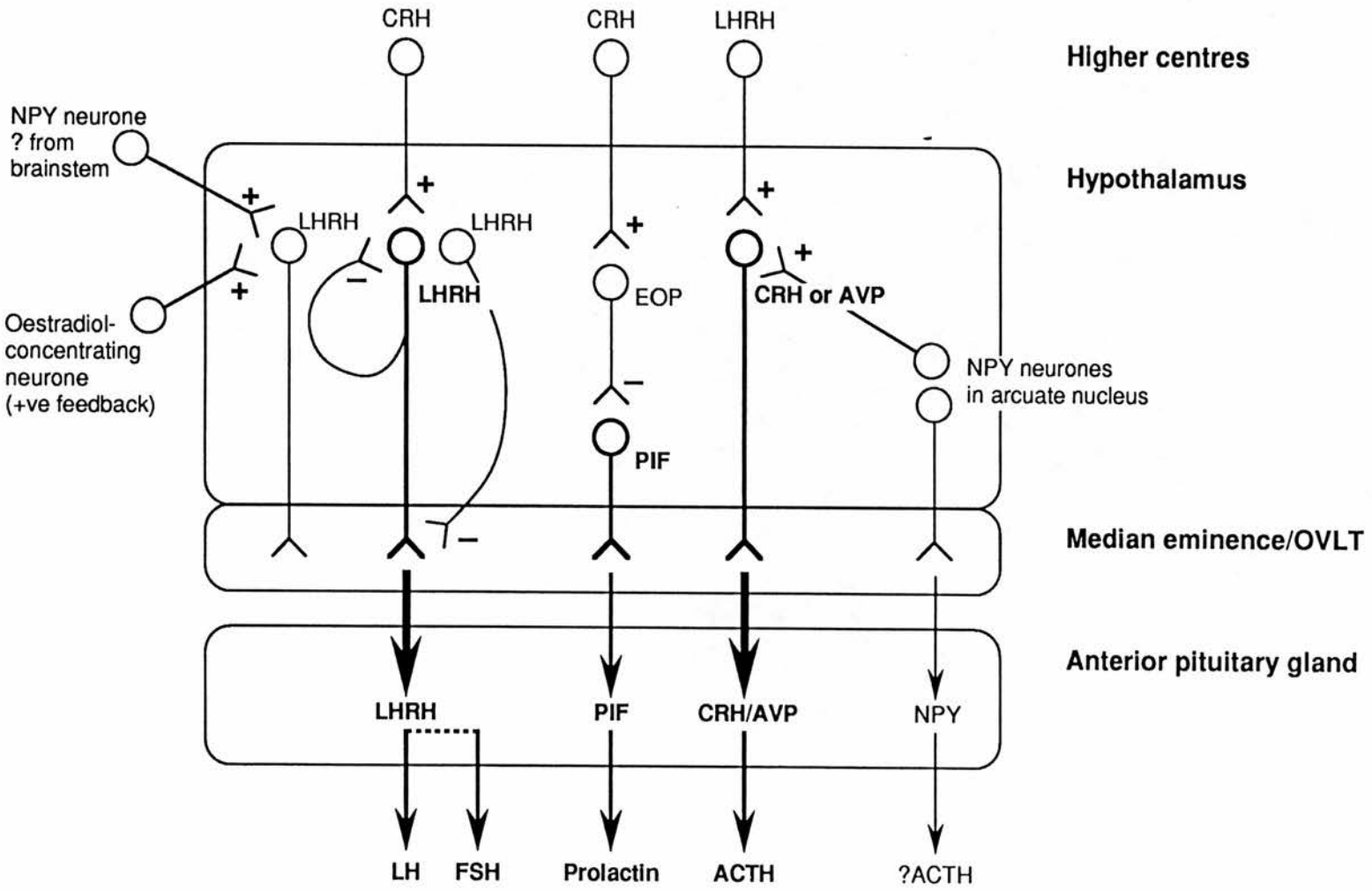
We also found differences between the responses to NPY in the sheep and those reported in the rat. Neither the experiments described in this thesis, nor the results of Brooks *et al.* (1990), indicate any pituitary locus of action for NPY in the regulation of reproduction in the sheep. This questions the suitability of the rat as a model for the ewe, or indeed for other species including man. Much care is required in the interpretation of results across species, particularly with respect to the hypothalamo-pituitary-adrenal axis (see Chapter 7). This was highlighted in a recent article by Funder (1990), in which the commonality and differences between animal models used in endocrinological studies was discussed and the 'normative' physiology of the rat brought into question.

The precise physiological roles of the mechanisms demonstrated by the current investigations remain somewhat speculative. However, it is possible to propose a hierarchy of neural circuitry involved in the

ation of LHRH, CRH and NPY pathways with respect to the luctive and hypothalamo-pituitary-adrenal axes. The secretion of ıd ACTH is clearly under the control of LHRH, and CRH and/or respectively, from the hypothalamus. Superimposed upon these ry controls is the influence of LHRH on its own secretion, the action H from higher centres on LHRH, and the modulation by NPY from ainstem and the arcuate nucleus on LHRH and CRH secretion (see 10.1).

e in-vivo approach used in the studies has many advantages and tended our knowledge of the action of hypothalamic neuropeptides. ver, it can only demonstrate the net, pituitary effect of all the ıs affected by the treatment applied. Even if we were able to sample blood, in this respect the hypothalamo-pituitary unit would still ole a 'black box'. A specific localization of effect could be achieved by of the microinjection of the peptide/antagonist into particular

Figure 10.1. Schematic diagram showing some of the interactions of LHRH, CRH and NPY in the regulation of the reproductive axis and of the hypothalamo-pituitary-adrenal axis. Primary control mechanisms are shown in bold. Hypothetical excitatory and inhibitory pathways are indicated. LHRH: luteinizing hormone-releasing hormone; CRH: corticotrophin-releasing hormone; NPY: neuropeptide Y; AVP: arginine vasopressin; EOP: endogenous opioid peptide; PIF: prolactin-inhibiting factor; ACTH: adrenocorticotrophic hormone, corticotrophin; LH: luteinizing hormone; FSH: follicle-stimulating hormone; OVLT: organum vasculosum lamina terminalis.


CRH
CRH
LHRH
Higher centres
NPY neurone
? from
brainstem
Hypothalamus
LHRH
LHRH
LHRH
EOP
CRH or AVP
Oestradiol-
concentrating
neurone
(+ve feedback)
NPY neurones
in arcuate nucleus
PIF
Median eminence/OVLT
Anterior pituitary gland
LHRH
PIF
CRH/AVP
NPY
LH
FSH
Prolactin
ACTH
?ACTH

nuclei or terminal fields, or by push-pull perfusion of individual brain areas. The latter technique has the disadvantage that less control is possible over the precise dose delivered. Tissue damage at the site of interest is also a major problem. In addition, the push-pull perfusion technique is more reliable with some neuropeptides than others (e.g., see Rondeel, de Greef, van der Waart *et al.* 1989). These approaches are more effective in species such as the rat, where the stereotaxic co-ordinates of specific nuclei are constant and predictable. However, the inter-animal variation is notably very wide in sheep, even within breeds, and this can cause considerable complications with the identification of intra-hypothalamic structures.

The experiments in this thesis have addressed only some of the neuropeptides potentially involved in the hypothalamic control of the secretion of hormones from the anterior pituitary gland. Other examples for investigation include CCK, which is a potent inhibitor of LH secretion in the OVX rat, and VIP which stimulates LH secretion both *in vivo* and *in vitro*. These and other candidates for the integrative control of the hypothalamo-pituitary unit have been discussed further by McCann, Samson, Aguila *et al.* (1986). In addition, two recently discovered neuropeptides worthy of further investigation in this regard are the hypothalamic peptide amides, galanin and PACAP (pituitary adenylate cyclase-activating polypeptide).

Galanin is a 29-amino acid peptide amide originally isolated from porcine intestine by Tatemoto, Rökaeus, Jörnvall *et al.* (1983). In the rat, ghest brain concentrations are found in the hypothalamus and ME ström, Melander, Hökfelt *et al.* 1987). Moreover, co-existence of n-ir with many of the classical neurotransmitters suggests that it unction as a modulator of neuroendocrine function (Melander, s, Hökfelt *et al.* 1985; Melander, Hökfelt, Rökaeus *et al.* 1986; Levin, enko, Howe *et al.* 1987). Although a physiological role for galanin ot been defined, it has been shown to influence the secretion of n hormone, prolactin and dopamine in man and rat, probably acting level of the hypothalamus (Bauer, Ginsberg, Venetikou *et al.* 1986; z, Samson & McCann, 1986; Melander, Fuxe, Härfstrand *et al.* 1987; tröm, Melander, Hökfelt *et al.* 1987). In addition, hypothalamic for galanin has been shown to increase after oestrogen treatment rat (Kaplan, Gabriel, Koenig *et al.* 1988). However, in another study, halamic levels of the peptide were unaltered by ovariectomy or

oestrogen treatment, though ovariectomy reduced pituitary galanin content considerably and oestrogen produced a significant increase in pituitary galanin and galanin mRNA content (O'Halloran, Jones, Steel *et al.* 1990). A role for galanin in the regulation of the reproductive axis is still speculative, and it is possible that it may have a hypothalamic function in addition to an autocrine or paracrine role at the pituitary level.

PACAP is a bioactive peptide isolated very recently from ovine hypothalamic tissue by Miyata, Arimura, Dahl *et al.* (1989). It was so-named because of its ability to stimulate the accumulation of intra- and extracellular cAMP in cultures of rat anterior pituitary cells. Elucidation of the primary structure of PACAP revealed it to be a 38-amino acid peptide amide of the VIP family (PACAP38). $PACAP_{(1-28)}$ shows 68% homology with porcine VIP; however, the $PACAP_{(29-38)}$ C-terminal region appears to be unique. PACAP was shown to contain an amidation site at amino acid positions 29–30, which suggested the presence of a shorter amidated peptide of 27 amino acid residues, PACAP27. This has also been isolated from ovine hypothalamus (Miyata, Katsuura, Gottschall *et al.* 1989). Dense immunoreactive fibre networks of both PACAP27 and PACAP38 have been demonstrated throughout the hypothalamus and septum of the sheep, and particularly in the ME; immunoreactive cell bodies were also demonstrated in the PVN and the supraoptic nucleus (Köves, Arimura, Somogyvári-Vigh *et al.* 1990). In addition, these authors demonstrated the presence of PACAP-ir fibres clinging to unstained cell bodies and their

ites in the lateral hypothalamus and lateral septum, and also nding blood vessels in the latter area. Thus it appears that PACAP lay a multifunctional role including that of a hypophysiotrophic ne, neurotransmitter, neuromodulator, and vasoregulator.

e current studies have shown clearly that neuropeptides have a functional role within the hypothalamus, acting as releasing factors, transmitters and neuromodulators. Further research, outlined needs to be done to explore the *specific* actions of LHRH, CRH and t particular intra-hypothalamic loci. This could include, in the first ce, the electrophysiological analysis of neuropeptide action both *in* nd *in vitro*, using isolated brain slice preparations (see Ferguson & d, 1987; Pittman, MacVicar & Colmers, 1987). This would allow the of hypothalamic function in more detail in the context of a specific nal circuit and at the membrane level. A first step could be to gate the concept of LHRH autoregulation or alternatively, the

hypothesis that NPY acts as a neurotransmitter within the PVN. Such an approach, either *in vivo* in the rat, or *in vitro*, would complement the findings of the current neuropharmacological studies.*

* Plans have been made to move to the Neuroscience Research Group at the University of Calgary, under the auspices of Dr. Quentin J. Pittman, where electrophysiological investigation of the role of NPY as a neurotransmitter in the rat PVN will be carried out *in vivo* and *in vitro*.

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